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PROCEEDINGS
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# *ANTIBIOTIC SUBSTANCES FROM BASIDIOMYCETES. X. FOMES JUNIPERINUS SCHRENK**

By Marjorie Anchel, Annette Hervey and William J. Robbins
The New York Botanical Garden and Department of Botany, Columbia University

Communicated June 25, 1952

In previous publications[1] from this laboratory, three isolations[2] of *Fomes juniperinus* were reported to evidence antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*.

Each of these isolations was grown in Fernbach flasks on a corn steep medium.[3] The culture liquids had antibacterial activity of 32 dilution units per ml. (as measured against *Staph. aureus*) in $2^1/_2$ months after inoculation, and 64 to 256 dilution units after 3 months or more. Supplementing the corn steep medium with 1.0 g. of fish solubles (Van Camp Laboratories) per liter did not improve the development of antibacterial activity. The corn steep medium without the Dox minerals was no more satisfactory than the complete medium. Omitting the sodium nitrate from the complete medium was detrimental, in contrast to the results obtained with the production of pleuromutilin by *P. mutilus*.[4] Shake cultures were unsuccessful because of the slow growth of the fungus.

Through the courtesy of Ross W. Davidson six additional strains[5] of *F. juniperinus* were obtained which were more active producers of antibacterial substances under our conditions. Some of these produce in 2 to $2^1/_2$ months culture liquids with 128 to 256 dilution units as measured against *Staph. aureus*. On reflooding the mats with fresh corn steep medium, culture liquids with an activity of 256 dilution units were obtained in three weeks. No correlation between vigor of growth of the various strains and antibiotic activity was noted.

*Isolation of a Crystalline Antibacterial Substance.*—A crystalline compound, which accounts for most of the antibacterial activity, was isolated from the culture liquid of eight of the strains of *F. juniperinus*. (Strain 72028-S was not used.) The compound, which has been named *fomecin A*, has the molecular formula $C_8H_8O_5$. At least one other antibacterial substance may be present in the culture liquid.

Antibacterial material was not readily extracted from the culture liquid by organic solvents although at low pH some extraction was accomplished by ethyl acetate. The active material was adsorbed quantitatively on Norit A and about 30 per cent was eluted by aqueous acetone.

The procedure used for the isolation of fomecin A was as follows: Batches of active culture liquid of about 20 liters were adjusted to pH 1 to 2 by the addition of concentrated HCl. The acidified solution was stirred with Norit A, 25 g. per liter, for half an hour and allowed to stand overnight in a cold room at about 7°C. The charcoal was filtered off and eluted three times with 80 per cent acetone using 10 per cent of the volume of the original culture liquid for the first elution and 5 per cent each time for the second and third elutions. The eluates were combined and the acetone removed under reduced pressure; the water bath was kept at from 50° to 60°C. The aqueous solution remaining (about 5 per cent of the volume of the original culture liquid) was subjected to a modified ten funnel counter-current distribution between water and ethyl acetate. The modification consisted in continuing the distribution after all ten funnels had been equilibrated, until all the water phases had passed through the tenth ethyl acetate phase. Most of the pigment remained in the aqueous phase and was carried through in the original water layer. Crude crystalline fomecin A was usually obtained from the ethyl acetate phase of the last six funnels. A yield of 0.36 g. of crude crystalline fomecin A was obtained per liter of culture fluid (Ochoco strain) which had an activity of 256 dilution units per milliliter as measured against *Staph. aureus*. This amount of crystals accounted for less than 10 per cent of the activity of the original culture liquid.

*Chemical Properties of Fomecin A.*—Crude crystalline fomecin A obtained from the counter-current distribution was crystallized from ethanol-water, ethanol-benzene, acetone-benzene or ethyl acetate. The color and form of the crystals varied strikingly in different recrystallizations. The color ranged from light cream or off-white to a bright orange. Different forms obtained were heavy hexagonal prisms, thin triangular plates, and rods or sharp needles. The various forms and colors seemed to be associated with the type of solvent (more or less polar) and with concentrations at the inception of crystallization. It was frequently possible to induce crystallization of a desired form by seeding with that form. Fomecin A was found to be a weakly acidic compound, sparingly soluble in water (roughly 1 mg. per ml.), slightly more in ethanol, acetone and ethyl acetate, less in chloroform and very slightly in benzene. The compound was stable to short boiling in aqueous solutions at neutral or acid pH. Above pH 8 the solution darkened and with a few seconds of boiling about half of the antibiotic activity was lost. In an open capillary or sealed in vacuum fomecin A did not melt but charred gradually above 160°C. Its solution in 95 per cent ethanol was optically inactive. It showed specific absorption in the ultra-

violet with maxima at 241 mμ (ε = 10,760) and 305 mμ (ε = 14,260). Studies which will be reported separately suggest a quinoid type of structure.

Two separate samples were used for analysis.[6] One (sample 1) was obtained from the culture liquid of *F. juniperinus* (CBS) and was recrystallized from ethyl acetate. The other (sample 2) was obtained from the culture liquid of strain Ochoco and was recrystallized three times from ethanol-water. Both samples were dried *in vacuo* at 78°C. for 3 hours.

| | C | H | O | NEUT. EQUIV. | MOL. WT. |
|---|---|---|---|---|---|
| Found: | | | | | |
| Sample 1 | 52.59 | 4.49 | ... | 173 | ... |
| Sample 2 | 52.28 | 4.49 | 43.47 | ... | 189 |
| Calcd. for $C_8H_8O_5$ with one acidic grouping | 52.18 | 4.38 | 43.44 | 184 | 184 |

*A Second Active Principle.*—A second antibacterial substance in the culture liquids and in the eluate is suspected on the following basis. The culture liquid and eluate were from 4 to 8 times as effective on *Staph. aureus* as on *Mycobacterium smegma*. Fomecin A was 32 times as active on *Staph. aureus* as on *Myco. smegma*. The activity on *Staph. aureus* of the first water extract passing through the counter-current system was from 2 times to $^1/_8$ that on *Myco. smegma*. It appears probable that a substance with greater relative activity on *Myco. smegma* than fomecin A accumulated in the water phase of the first tubes of the counter-current distribution.

It is possible to follow fomecin A in the counter-current distribution by the ultra-violet absorption peak at about 300 mμ. The first one to four water extracts passing through the counter-current system show a peak at 260–270 mμ and are actively antibacterial. It is not yet clear whether the ultra-violet absorption peak at 260–270 mμ belongs to a hypothetical fomecin B or whether it is associated with a compound which is neither fomecin A nor B.

*Antibacterial Activity of Fomecin A.*—The antibacterial activity of fomecin A expressed as the minimum concentration in micrograms per milliter that inhibited the growth of bacteria for 24 hours (Mycobacterium 42 hours) was as follows:

ANTIBACTERIAL ACTIVITY

| | |
|---|---|
| *Staphylococcus aureus* | 8–16 |
| *Bacillus mycoides* | 250 |
| *Bacillus subtilis* | 31–63 |
| *Mycobacterium smegma* | 250–500 |
| *Escherichia coli* | 125 |
| *Klebsiella pneumoniae* | 63 |
| *Pseudomonas aeruginosa* | 125 |

*Antifungal Activity.*—Antifungal activity of fomecin A was determined by serial dilution in a peptone medium at pH 6.0 using spore suspensions of fungi as inoculum.[7] Trichophyton was incubated at 30°C., the others at 25°C. The compound had little activity on the fungi tested. The minimum inhibitory concentration of fomecin A was 500 μg. per ml. for *Trichophyton mentagrophytes*. The growth of the following fungi was not inhibited by as much as 500 μg. per ml.: *Aspergillus niger*, *Chaetomium globosum* (USDA 1042.4), *Gliomastix convoluta* (PQMD4c), *Memnoniella echinata* (PQMD1c), *Myrothecium verrucaria* (USDA 1334.2), *Penicillium notatum* (832), *Phycomyces blakesleeanus* (+ strain), *Saccharomyces cerevisiae* (188) and *Stemphylium consortiale* (PQMD 41b).

*Antiviral Activity.*—Fomecin A was tested by the paper disc method[8] for activity against 59 bacteriophages and one actinophage (*Streptomyces griseus*). Inhibition of phage activity was observed on one of four streptococcal phages, one of six enterococcal phages, three of sixteen phages of *E. coli*, two phages of *Salmonella schottmuelleri*, one page of *Eberthella typhosa*, seven of eleven phages of *Vibrio cholerae* and on the actinophage.

*Activity in Presence of Blood.*—Incubation at 37°C. for 4 hours with 5% whole human blood in beef extract broth reduced the activity of fomecin A from 64 dilution units to 2 dilution units.

*Animal Toxicity.*—In preliminary tests using Carworth male white mice weighing on an average 18–20 g., intravenous injection of fomecin A in doses up to 1 mg. per mouse (ca. 50 mg. per kilo) had no apparent ill effect. Five groups of mice, six in a group, were used for the tests. All mice were treated by intravenous injection into a tail vein. One group, used as control, received 0.5 ml. of 0.9 per cent saline. Groups two to five received the same volume of saline containing 1.0, 0.5, 0.25 and 0.1 mg. of fomecin A, respectively (the solutions were adjusted to pH 7.6 with sodium hydroxide). After one week all mice appeared normal.

* This investigation was supported in part by grants from the National Microbiological Institute, Federal Security Agency, Public Health Service and from the Charles Pfizer Company. The authors also acknowledge the technical assistance of Marvin Cohen and Alice Hesse.

[1] Robbins, W. J., Hervey, Annette, Davidson, R. W., Ma, Roberta, and Robbins, W. C., *Bull. Torrey Bot. Club*, **72**, 165–190 (1945). Hervey, Annette, *Ibid.*, **74**, 476–503 (1947).

[2] Two of these, 72028-S and CBS, were obtained from R. W. Davidson. The third, F-3765, was furnished by Dr. Mildred K. Nobles and came originally from Baarn, Holland. It is possible that strains CBS and F-3765 are from the same isolation.

[3] The complete medium contained per liter, 1.5 g. $KH_2PO_4$, 0.5 g. KCl, 0.5 g. $MgSO_4 \cdot 7H_2O$, 3 g. $NaNO_3$, 40 g. dextrose and 5 g. Staley special nutrient 114 (corn steep).

[4] Kavanagh, F., Hervey, Annette, and Robbins, W. J., these PROCEEDINGS, **37**, 570–574 (1951).

[5] These strains were Whitman A, B and C, from sporophores on Juniperus, Whitman National Forest, Oregon, 1950. Ochoco: From *Juniperus occidentalis*. Ochoco Na-

tional Forest, Oregon, Collected by Childs, 1950. 66286: From sporophore on *Juniperus utahensis*, Mesa Verde National Park, Colorado, 1944. 94141: From *Juniperus occidentalis*, City Park Bend, Oregon, 1941. The Ochoco strain was the best for the production of antibacterial culture liquids.

[6] The analysis of sample 1 was made by J. F. Alicino and of sample 2 by the Huffman Analytical Laboratories. The molecular weight was determined by the ebullioscopic method.

[7] Kavanagh, F., Hervey, Annette, and Robbins, W. J., these PROCEEDINGS, **35**, 343–349 (1949).

[8] Asheshov, I. N., Strelitz, Frieda, and Hall, Elizabeth A., *Brit. J. Exp. Path.*, **30**, 175–185 (1949). The authors are indebted to Dr. Asheshov for the tests on antiphage activity.

## *SEXUALITY IN THE ACRASIALES**

BY CHARLES M. WILSON

THE BIOLOGICAL LABORATORIES, HARVARD UNIVERSITY

Communicated by Kenneth B. Raper, June 9, 1952

Although there has been an increasing use of members of the Acrasiales as experimental organisms in morphogenetic and physiological research and a wider use of these primitive slime molds for laboratory study in the past few years, the cytological aspects of the life cycles of these organisms were not known. With this situation in mind and knowing the great need of a critical cytological study of the genus Dictyostelium, the author began the present work in the spring of 1951 with the encouragement of Dr. Ralph Emerson, who supplied a culture of *Dictyostelium discoideum* Raper which he had in turn received from Raper.

Earlier works have revealed little or nothing concerning the nuclear behavior in the Acrasiales. It has been generally assumed that the Acrasiales lack sexuality. Bonner,[1] one of the present day researchers on the group, notes that it is accepted today that the Acrasiales differ from the Myxogastrales in lacking sexuality; he also states that Skupienski[2] is the only investigator who does not agree with this concept and that his view is now generally discredited. Skupienski described the pairing and fusion of gametic myxamoebae and the formation of the aggregation by zygotes in *Dictyostelium mucoroides*. He reported a double division by the cells that went into the formation of the sporangium and postulated that this was comparable to tetrad formation in other cryptogams. He stated, however, that it was not possible to clarify the problem because of inadequate methods of fixing and staining at the time. The lack of conclusive proof prevented acceptance of his observations. K. B. Raper, who initiated the significant modern research on Dictyostelium, does not in his numerous

papers on the simple slime molds consider Skupienski's report of sexuality in Dictyostelium valid enough for acceptance. Kniep[3] reviewed the investigations on sexuality in the Myxogastrales and the Acrasiales, including the work of Skupienski, and recognized that more cytological work was needed on these organisms before conclusive statements could be made concerning their life cycles. Although some progress has been made in understanding the cytology of the Myxogastrales, the present status of the Acrasiales remains as described above.

For the present investigation *Dictyostelium discoideum* was grown as a two-membered culture with *Escherichia coli* after the manner described by Raper.[4] The medium used was yeast-starch agar.[5] The morphology of the stages of development was described by Raper[6] in his original description of the species, and his terminology will be used here. With current methods of staining, it has been possible in the present work to study cytologically the most important periods in the life cycle of Dictyostelium. At various stages in the life cycle samples were removed from culture dishes and smeared with aceto-orcein.

*Vegetative Stage.*—The myxamoebae, which have emerged from spores, feed upon the bacteria and by cell division greatly increase in number The cells have large food vacuoles containing bacteria which are readily stained with aceto-orcein.

*Aggregation Stage.*—When a sample is collected and smeared of the very early stages of aggregation, vegetative amoebae, fusing gametic amoebae and zygotes are found. The cells initiating the aggregation stage are gametic. Although fusion of gametes is rapid and difficult to find, it has been found that syngamy occurs on the outer fringes of the aggregation and that fusion is accomplished by one gamete enveloping another with pseudopods. The zygotes form small masses in the culture dish, and as other zygotes are formed, they stream into these masses.

Meiosis, as well as syngamy, occurs at the aggregation stage. After the first formed zygotes have created a center of the aggregation, the reduction divisions begin and take place as long as zygotes continue to join the aggregation. The nuclear divisions are not simultaneous. This is not surprising when it is remembered that in aggregation, as in all of the other stages, each cell is an entity and develops at a rate of its own, within certain limitations. In fact, some divisions can be observed in the pseudoplasmodium after it has begun to migrate. These divisions are probably Meiosis II in lagging zygotes, but the possibility of scattered mitotic divisions after meiosis must not be precluded. In *Dictyostelium discoideum* the haploid number of chromosomes is seven. The zygotes are larger than either the vegetative cells before syngamy or the cells after the reduction divisions. Even under low magnification it is possible to distinguish the

zygotes because of their size. Since the fusion nucleus of the zygote is the only diploid nucleus in the life cycle, there are no mitotic divisions in the diploid stage. This is an important difference from the Myxogastrales in which many simultaneous divisions take place in the diploid nuclei of the plasmodium. Aggregation, which is the expression of the diplophase in Dictyostelium, is initiated by the sexual phenomenon of syngamy and ended after meiosis.

*Migration Stage.*—The pseudoplasmodium is endowed with a certain amount of mobility which permits it to reach a favorable position for fruiting. *D. discoideum* is the only member of the genus described as having this stage,[6] but the slime track left by the pseudoplasmodium with amoebae scattered in it is similar to the slime envelope which surrounds the long attenuated stalks formed by other members, such as *D. purpureum*. Until the stalk of *D. purpureum* ceases elongation, the mass at its tip is very similar to the pseudoplasmodium of *D. discoideum*. This has been indicated by Raper.[7]

*Sorocarp Formation.*—The gross morphology of the sorocarp has been well described by Raper and Bonner. Bonner,[1] Bonner and Eldredge,[9] Bonner and Slifkin,[10] Olive[11] and Raper[8] have stated that there is no increase in cell number after the vegetative stage. Bonner has evidently confused cell division and growth. He states, "In order to fully understand these results the reader must be reminded that from the moment the cells of Dictyostelium come together to form a pseudoplasmodium there is no cell division, no growth."[10] It can be agreed that there is no growth in Dictyostelium after aggregation, if one defines growth as assimilation and increase in size or mass, for the myxamoebae cease feeding prior to aggregation. For Bonner to have assumed that lack of assimilation excluded cell division is erroneous. The production of reproductive cells, either vegetative or sexual, often, in the algae, fungi and other plants, entails merely the increase in number of cells and reduction of cell size. Indeed, the reduction of cell size and increase in numbers afford great advantages in dispersal. It has been found in the present study of Dictyostelium that after the stalk of the sorocarp has elevated the sporogenous cells, rapid cell division takes place before the formation of spores. This division of sporogenous cells causes a reduction in size of the cells which are to become spores. Occasionally large binucleate spores can be found, and these indicate that the spore was formed after nuclear division but before cell division, or at least that the spore was formed before the last cleavage of the cytoplasm occurred.

This work points out two important periods of cell division after the vegetative stage, the meiotic divisions at aggregation and the mitotic divisions at spore formation. The author confirms Skupienski's observation of fusing gametes and goes further in revealing the position of meiosis in the

life cycle of Dictyostelium and in revealing other nuclear behavior at certain critical points in the life cycle.

* Part of this work was done as a National Research Council Fellow at the University of California, Berkeley.

[1] Bonner, J. T., *Am. J. Bot.*, **31**, 175 (1944).

[2] Skupienski, F. X., *Recherches Sur Le Cycle Evolutif Des Certains Myxomycetes*, Paris, 1920.

[3] Kniep, H., *Die Sexualität der niederen Pflanzen*, Gustav Fischer, Jena, 1928.

[4] Raper, K. B., *The Quarterly Review of Biology*, **26**, 169 (1951).

[5] Emerson, R., *Lloydia*, **4**, 77 (1941).

[6] Raper, K. B., *J. Agr. Res.*, **50**, 135 (1935).

[7] Raper, K. B., *Am. J. Bot.*, **27**, 436 (1940).

[8] Raper, K. B., *Growth, The Third Growth Symposium*, **5**, 41 (1941).

[9] Bonner, J. T., and Eldredge, D., *Growth*, **9**, 287 (1945).

[10] Bonner, J. T., and Slifkin, M. K., *Am. J. Bot.*, **36**, 727 (1949).

[11] Olive, E. W., *Boston Soc. Nat. Hist. Proc.*, **30**, 451 (1902).

## *A REVERSIBLE PHOTOREACTION CONTROLLING SEED GERMINATION*

H. A. BORTHWICK, S. B. HENDRICKS, M. W. PARKER, E. H. TOOLE AND VIVIAN K. TOOLE

BUREAU OF PLANT INDUSTRY, SOILS AND AGRICULTURAL ENGINEERING, BELTSVILLE, MARYLAND

Communicated June 7, 1952

Effect of light upon germination of some seed is one manifestation of a very general, possibly universal, phenomenon controlling living processes. Some of the details of this effect on lettuce seed (*Lactuca sativa* L.) are given here. Their bearing on the nature of a *reversible* photoreaction involved is developed.

Germination of one variety of lettuce seed was found by Flint and McAlister[1] and Flint[2] to be promoted by radiation in the region of 5250 to 7000 Å. The greatest response for promotion resulting from a given irradiance was in the region of 6600 Å. Germination was inhibited by radiation in the region of 7000 to 8200 Å. with the maximum inhibition between 7100 and 7500 Å. We have verified the observations of Flint and McAlister for wave-lengths greater than 5200 Å. and have quantitatively determined the action spectrum (Fig. 1).

The action spectrum is expressed in terms of the incident energy required at various wave-lengths to promote or to inhibit germination to half its maximum value. The results were obtained with a two-prism (glass) spectrograph having a dispersion of about 35 Å./cm. at 6000 Å.[3] The slit

width was about 90 Å. at this wave-length and the incident energy was 0.100 $\times 10^{-3}$ joules/cm.$^2$ sec. Lettuce seeds for these experiments were placed on wetted blotters in dishes that were immediately covered and maintained in darkness at 20°C. for various times before removal for irradiation. Those to be inhibited in germination were first exposed to radiation between 5800 and 6600 Å., as obtained with filters and a 4500° white fluorescent source. The exposure was such as to lead to germination of more than 98% of the viable seeds in controls. Following this treatment the seeds were irradiated in various wave-length regions to test for inhibition of germination. Those to be promoted were taken directly from darkness and irradiated. After irradiation, all seeds were held for two days at 20° in darkness and the ones then germinated were counted.

Two pigments apparently are involved with other reactants in the action of light on germination. Evidence will be given that these materials are directly related by the reactions.

$$(1)\quad \underset{\substack{6500\ \text{Max.}\\ \text{Dormant}}}{\text{Pigment} + \text{RX}} \underset{\text{Infra Red}}{\overset{\text{Red}}{\rightleftharpoons}} \underset{\substack{7300\ \text{Max.}\\ \text{Germinating}}}{\text{Pigment X} + \text{R}}$$

$$(2)\quad \text{Pigment X} + \text{R} \underset{\text{Infra Red}}{\overset{\text{Red}}{\rightleftharpoons}} \text{Pigment} + \text{RX}$$

$$(3)\quad \text{with (1)}\ \text{Sub.} + \text{RX} \rightarrow \text{R} + \text{Sub. X}$$

$$(4)\quad \text{with (2)}\ \text{Sub. X} + \text{R} \rightarrow \text{RX} + \text{Sub.}$$

Two equations are given since no entirely trustworthy method has been found to associate Pigment and Pigment X with their respective absorption maxima. The nature of the pigment and the possibility of other reactants will not be examined since we do not wish to obscure the immediate deduction of a reversible photoreaction with any speculation.

The sample of Grand Rapids lettuce seed used was equally sensitive to promotion by red radiation for 4 to 16 hr. after imbibition. Fifty per cent germination required 2.11 $\times 10^{-3}$ joules/cm.$^2$ of incident energy between 6530 and 6635 Å. and 10.1 $\times 10^{-3}$ joules/cm.$^2$ gave 90 per cent germination with a linear variation of the probit with log energy. Seeds that had been exposed to red radiation were equally sensitive to suppression of germination by radiation at wave lengths greater than 7000 Å. over this period of 4 to 16 hr. After 16 hr. of imbibition, the radiant energy required for promotion to a given per cent germination increased and after 31 hr. had changed by about threefold. During this period, the requirement for inhibition decreased by about the same amount. This was the first indication of a relation between the photoreceptive pigments. It probably arises, however, from a thermal conversion of RX or R toward the condition on the right side of the equations.

The dependence of the germination response solely upon the last of a

sequence of exposures to red and infra-red radiation is striking evidence of the immediate formation of the one pigment from the other. Seven lots of 200 lettuce seed were exposed to red radiation (5800 to 6800 Å.) for one minute after 16 hr. of imbibition of water in darkness. One lot was removed to the dark and the remaining ones were exposed to infra-red radiation of wave-lengths longer than 7000 Å. for four minutes. Again one lot was removed to the dark and the rest were exposed to red radiation for one minute. The alternations of red and infra-red irradiations were continued until all lots were treated. After this the treated and control lots of seed were held in darkness at 20°C. for two days. The germinated seeds were then counted (table 1).

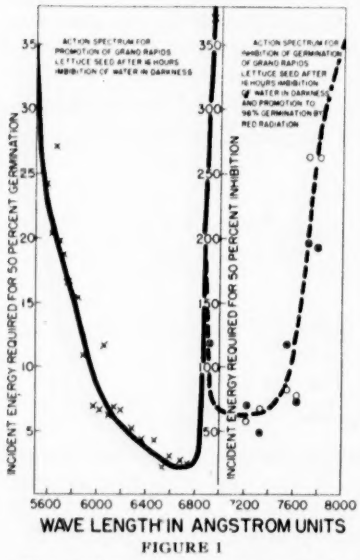


FIGURE 1

The action spectrum for promotion (solid line, ordinate on the left) and inhibition (dotted line, ordinate on the right) for germination of Grand Rapids lettuce seed. The several symbols give results from different experiments. Ordinates are in joules $\times 10^{-3}/cm^2$.

Energy greatly in excess of that required for full germination does not appreciably influence the sensitivity to inhibition by infra-red radiation. Thus a thirty-two fold increase in irradiance between 5800 and 6800 Å. over that required for 98 per cent germination of seed soaked for 16 hr. raised by less than twofold the energy required in the infra-red for suppressing the germination to a particular value. The result is in harmony with displacement of the equilibrium in a photoreaction and indicates that the pigment does not serve merely to transfer energy. Rather a certain energy is absorbed to activate the change of the pigment from one form to another.

The increased energy required for a given per cent germination, as the time of imbibition prior to irradiation is prolonged to periods greater than 16 hr., indicates that RX in equation (1) or R in equation (2) is limiting. In other words after long periods of imbibition, energy absorbed by the pigment is dissipated in some way, such as fluorescence, before reaction (1) or (2) takes place. That the pigment or pigment X is not limiting is shown by the simultaneous decrease in the energy required for inhibition. In fact this change in response is one evidence that a reactant other than the pigment is involved in the photoreaction.

After displacement by radiation does the reaction return toward an equilibrium in darkness? Experiments to answer this question must avoid confounding the sequence of germination following the displacement of the reaction to the right with a possible back reaction having about the same time constant. Seed of Grand Rapids lettuce was held fully imbibed at 20°C. for 16 hr. and then irradiated with energy adequate for full promotion of germination. This treatment was followed 7 hr. later by irradiation in the infra-red adequate to inhibit germination fully when given immediately after promotion. About 50 per cent of the seeds germinated. This indicates that in 7 hr. the germination process in half the promoted seeds had proceeded beyond determination by light.

An answer to the question was afforded by germination of a white-seeded lettuce variety that was thought to be more sensitive to temperature than Grand Rapids. Seeds of this variety germinate in light or in darkness at 20°C. but are dormant at 30°. After having been held for several days in

TABLE 1

LETTUCE SEED GERMINATING IN POPULATIONS OF 200 AFTER EXPOSURE TO RED AND INFRA-RED (IR) RADIATION IN SEQUENCE

| IRRADIATION | GERMINATION, % |
|---|---|
| None (dark control) | 8.5 |
| Red | 98 |
| Red + IR | 54 |
| Red + IR + Red | 100 |
| Red + IR + Red + IR | 43 |
| Red + IR + Red + IR + Red | 99 |
| Red + IR + Red + IR + Red + IR | 54 |
| Red + IR + Red + IR + Red + IR + Red | 98 |

darkness at 30° these seeds remain dormant if returned to 20° in darkness. However, if they are first irradiated in the region 5800 to 6800 Å. before returning to 20° in darkness, full germination is promoted. It follows that the reaction for the pigment was displaced from right to left in darkness at 30°. Existence of such a dark reaction indicates that the radiant energy is chiefly required for pigment activation rather than for the free energy change in the reaction.

The reaction can be poised at any point for a time adequate for germination; that is, a photochemical steady state can be established. The per cent of germination depends upon the integrated absorption of the two pigments. Thus in some wave band between 6900 and 7100 Å., 50 per cent germination is effected by continuous irradiation.

We have not thoroughly studied the action spectrum in the region below 5200 Å. An irradiance of $90 \times 10^{-3}$ joules/cm.$^2$ in the region of 4600 Å. resulted in about 60 per cent germination of Grand Rapids lettuce seed that had been held imbibed for 16 hr. in darkness. Less than $0.75 \times 10^{-2}$

joules/cm.$^2$ gave 50 per cent germination. This indicates that blue radiation can be very effective for promotion of germination. Failure of promotion to full germination as well as absence of an inhibitory action of blue radiation on seed that had been irradiated in the red adequate for full germination requires more detailed study for understanding.

The immediate general extension of these observations is to the varied response of seed germination to radiation.[4, 5] Germination of some seed is promoted by continuous light in the visible,[6] while that of others such as *Phacelia tanacetifolia* Benth. and *Amaranthus caudatus* L. is inhibited.[7] This is a result of the integrated effect of the photoreaction and suggests that the amount of the pigments and reactants R and RX vary among seeds. Many seeds that apparently are insensitive to control of germination by light nevertheless have the reaction for control. Thus the white-seeded lettuce variety previously mentioned germinates under radiation from an incandescent-filament lamp or in darkness but fails when exposed to radiation having wave-lengths in the region of 7000 to 8000 Å.

Dormancy in a seed is a suspended animation for the small plant that is present. Dormant imbibed seeds are known to have been viable for at least 70 years.[8] In these cases the time is so great as to raise serious question about maintenance of organization for future germination by slow but continued expenditure of energy in respiration. Imbibed seeds that have been held for long periods in darkness germinated after they were finally examined in light. If, following examination, the seeds had been irradiated in the region from 7000 to 8000 Å, the dormancy would likely have been reestablished. This, for seeds, is the control of the living process that was mentioned in the first paragraph.

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# ABSOLUTE RATE THEORY FOR ISOLATED SYSTEMS AND THE MASS SPECTRA OF POLYATOMIC MOLECULES*

BY H. M. ROSENSTOCK,† M. B. WALLENSTEIN,‡ A. L. WAHRHAFTIG AND HENRY EYRING

CHEMISTRY DEPARTMENT, UNIVERSITY OF UTAH

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Studies of the interaction of electrons with atoms and molecules have been of fundamental importance in the development of present-day physical theories. As has been stated so many times, the general problem is now solved "in principle" by the quantum mechanics, but the application of the theory to only the simplest systems has been practicable.[1, 2] In recent years a very large amount of data has been accumulated on the "mass spectra" resulting from the bombardment of molecules by electrons of energy 50 and 70 volts.[3] There is also a significant amount of data on special aspects of the mass spectra of a few polyatomic molecules. A reasonably exact analysis of a mass spectrum would require detailed knowledge of all the electronic states both of the molecule and of all the ions formed from it by removal of electrons and by removal and rearrangement of nuclei. In this paper we present a statistical approach to the problem. Necessarily, assumptions and approximations are required. While this theory is in no way complete, we believe it permits the discussion and coordination of a large amount of the available data in terms of the structure of molecules and ions.

Knowledge of the effect of low-voltage electron bombardment on molecules, besides being of interest in its own right, has varied applications. It has bearing on the relation of molecular structure to chemical reactivity. It is well known that the major fraction of the effect of high energy radiation on matter, including living systems, is due to the low energy secondary electrons.[4] The rate theory presented here should also be applicable to other problems involving isolated systems having a large but finite number of degrees of freedom.

The ionization and dissociation of diatomic molecules by electron impact has by now become quite well understood. Hagstrum[5] has recently discussed in great detail the mass spectra of a number of diatomic molecules, explaining the formation of the several ions and their kinetic energies in terms of Franck-Condon transitions to the various electronic states of the diatomic ions. Another discussion is that of Stevenson[6] who calculated the relative abundance ratios $H^+/H_2^+$ and $D^+/D_2^+$ in the mass spectra of hydrogen and deuterium, again using the picture of Franck-Condon transitions to known electronic states. While any discussion of large polyatomic molecules mass spectra must be in accord with these discussions, the direct application of the same methods is impossible.

We start with the following hypothesis, first stated by us at the Oberlin Symposium on Biological Effects of Radiation in 1950 and later elaborated by Wallenstein:[7,8]

Following vertical ionization, the molecule-ion has a certain amount of excitation energy in its electronic and vibrational degrees of freedom, referred to the minimum of its lowest electronic state. Most of the time, this excited molecule-ion does not decompose immediately into ionized and neutral fragments but rather undergoes at least several vibrations. During these vibrations there is high probability of radiationless transitions among the many potential surfaces for the molecule-ion, resulting in a distribution of the excitation energy in completely random fashion. The molecule-ion decomposes only when the nuclei are in the proper configuration and a sufficient amount of vibrational energy has concentrated in the necessary degrees of freedom. The fragments in turn may have sufficient energy to decompose through a similar sequence of events. Rearrangement of the bonds, without dissociation, also may occur in the same fashion.

This hypothesis is based on the early work of Franck and coworkers.[9] It is implicit in the calculations of Stevenson and others on bond energies

TABLE 1

| MOLECULE | $\frac{\text{C–H BONDS}}{\text{TOTAL BONDS}}$ | $\frac{C_nH_{2n+1}^{+}}{\text{TOTAL IONIZATION}}$ [a] |
|---|---|---|
| Propane | 0.8 | 0.08 |
| *n*-Butane | 0.77 | 0.008 |
| *n*-Heptane | 0.73 | 0.00002 |

[a] Calcd. from data in reference 13.

for the processes involved in simple dissociation of the parent molecule-ion at electron voltages near the appearance potential.[10, 11] Similar considerations are also implied in a paper by Langer.[12] The extension of this idea of successive, quasi-equilibrium, unimolecular dissociations to the entire mass spectrum of a molecule is, we believe, new. It is not the complete story; certainly some ionization, especially multiple ionization, will yield immediate dissociation of a non-statistical character. The evidence is that such immediate dissociation follows only a small fraction of the total ionizing collisions.

Let us consider some of the arguments favoring the above hypothesis.

On the contrary assumption that the decomposition of the parent molecule-ion is determined by the location of a particular bond from which the electron is removed, a simple relation should exist between the relative abundance of certain product ions and the number of bonds available in the parent molecule-ion for this type of dissociation. For example, the relative amount of $C_nH_{2n+1}^{+}$ ions to total ions in a paraffin hydrocarbon mass spectrum should be proportional to the ratio of C–H bonds to total bonds. This is not the case, as may be seen from the data in table 1.

Further interesting evidence is obtained from the "total ionization" of a compound, i.e., the total amount of ions formed by a given electron current passing through the compound under specified conditions. There is a tremendous variation in the breakdown patterns among various isomers of a given composition, but the total ionization remains remarkably constant, as shown in table 2. This implies that the probability of removal of a va-

TABLE 2

| HYDROCARBON | NUMBER OF ISOMERS | TOTAL IONIZATION (ARBITRARY UNITS) MEAN VALUE | MAXIMUM DEVIATION, % | MEAN DEVIATION, % |
|---|---|---|---|---|
| $C_8H_{18}$ | 18 | 1.79 | 10 | $3^1/_2$ |
| $C_9H_{20}$ | 35 | 1.94 | 20 | $6^1/_2$ |
| $C_5H_8$ | 10[a] | 1.03 | 7 | 4 |

[a] Including cyclo-olefins, -dienes, and -ynes.

lence electron is essentially independent of the orbital occupied by the electron. The data on stopping power for high energy particles lead to the same conclusion.[17] However, different orbitals will contribute in widely different fashion to the strength of various bonds. Hence, we conclude that after formation but before dissociation of the ion there must be a period during which the energy is distributed throughout the ion.

For the postulated mechanism of energy transfer it is necessary that the density of electronic states for the ion be such that there exist sufficient crossings of the potential surfaces. Consider the propane ion which has nineteen bonding electrons. The number of states corresponding to this system of nineteen electrons all in their ground states is $2^{19}$.[1] However, many of these states are degenerate. The number of different eigenvalues, aside from accidental degeneracy, is

$$\frac{n!}{\left(\frac{n+1}{2}\right)!\left(\frac{n-1}{2}\right)!}$$

which for $n = 19$ is of the order of $10^5$. These distinct eigenvalues all lie in the range between zero, taken as the lowest state of the ion, and about 100 electron volts, corresponding to the completely antibonding state of the ion. This gives an average spacing between states of about one millivolt. There is, in addition, a still larger number of states corresponding to the various excited states of the separated atoms lying within the same limits. The vibrational energy of such a molecule-ion at room temperature is of the order of one-half electron volt, many times larger than the spacing between states. As the potential surfaces will differ greatly in their dependence on nuclear coordinates, the necessary number of crossings will surely exist.

A consequence of molecular-orbital theory for polyatomic molecules is that a molecule or ion with a half-filled orbital is better described in terms of non-localized molecular orbitals while a molecule containing paired electrons can be described either by non-localized molecular orbitals or by localized valence-orbitals.[18, 19] Thus, a system minus one electron will, in general, have not one much weakened bond but several bonds each weakened to a lesser extent. In particular, the totality of electronic states will not divide into two groups, one completely attractive, the other strongly repulsive in at least one bond direction. Rather, there will be many states which are attractive even though several antibonding electrons are present. In agreement with this, mass spectra show little evidence of fragments arising from strongly repulsive states and so having energies of several volts. This follows from direct measurements[20] and from the symmetrical shape of the mass peaks. Also since mass spectrometers strongly discriminate against ions having appreciable kinetic energy, the constancy of total ionization for isomers indicates that most ions are formed without appreciable kinetic energy.

A most serious gap in any theoretical discussion of mass spectra centers about the problem of energy transfer. One would like to have a distribution function for the fractions of parent ions formed with various amounts of internal excitation energy as a result of electron impact. An attack has been made on this problem only for the hydrogen and helium atoms.[2] However, if one can calculate the breakdown kinetics for parent ions having various internal excitation energies, it will be possible to determine an initial distribution function such that the observed mass spectrum is obtained. Such a distribution function should change in a simple manner for a series of electronically similar molecules such as the paraffin hydrocarbons.

A rate equation applicable to isolated systems may be developed in a fashion analogous to the absolute reaction rate theory of Eyring.[21] Other rate equations using similar reasoning have been discussed by Rice, Ramsperger and Kassel[22] and very recently by Marcus.[23] The liquid drop theory of the nucleus can likewise be treated in principle by this same method.[24]

The behavior of an isolated system is properly described in terms of a microcanonical ensemble,[25] with systems uniformly distributed over all states having energies in the region $E$ to $E + \delta E$ and having nuclear configurations corresponding to the initial chemical structure of the systems. For a reaction to occur, the system must have at least one other stable configuration; since initially the system has a known configuration, each member system of the ensemble can be described as a superposition of many pure states. Consistent with the previously stated hypothesis, it is assumed that the rate of transition of a system among the states is sufficiently

rapid so that the reaction yielding a different configuration has only a negligible effect on the distribution of the unreacted systems among the states. In general, it will be possible to select a "reaction coordinate," and an "activated complex" defined in terms of a saddle point on a potential surface, such that the change in the reaction coordinate describing the motion of the system through the saddle can be taken to be a translational motion.

In principle, the number of states of the system having energy between $E$ and $E + \delta E$, with the system in the initial configuration, can be calculated. If the number of states is large, this number can be represented by a density function: $\rho(E)\delta E$. The activated state will be defined by a total energy between $E$ and $E + \delta E$, with potential energy $\epsilon_0$ and kinetic energy between $\epsilon_t$ and $\epsilon_t + \delta\epsilon_t$ in the reaction coordinate. The corresponding number of activated states will be given by $\rho^{\ddagger}(E, \epsilon_0, \epsilon_t)\rho_t(\epsilon_t)\delta E\delta\epsilon_t$. Using the usual expression for translational energy, the density of translational states per length $l$ in the reaction coordinate is

$$\rho_t(\epsilon_t) = \frac{l}{h}\sqrt{\frac{2\mu}{\epsilon_t}}, \tag{1}$$

where $\mu$ is the "reduced mass" for the translation. The frequency of crossing the barrier, with $v$ the velocity along the reaction coordinate, is given by

$$\frac{v}{l} = \frac{1}{l}\sqrt{\frac{2\epsilon_t}{\mu}}. \tag{2}$$

Then the unimolecular rate constant is given by one-half the ratio of activated complexes to normal molecule-ions, multiplied by the frequency of crossing the barrier and integrated over all possible values of the translational energy in the reaction coordinate. The factor one-half arises from the equal probability of translation in opposite directions at equilibrium.

$$k = \int_0^{E-\epsilon_0} \frac{\rho^{\ddagger}(E, \epsilon_0, \epsilon_t)}{\rho(E)} \cdot \frac{1}{h}\, d\epsilon_t. \tag{3}$$

The description of the system as undergoing rapid radiationless transitions between electronic states could be replaced by a more general discussion in which no separation of electronic and nuclear motions is assumed except for the reaction coordinate. Equation (3) is consistent with either view-point.

In the applications which follow, state density functions are calculated for internal rotations and vibrations only. The electronic state density functions are assumed to cancel out. Nuclear motions are taken to be the same in all electronic states of a given ion or activated complex. Making the extreme idealization, the molecule a collection of $N$ weakly coupled harmonic oscillators, the activated complex a collection of $N - 1$ such oscillators, and using the Dirichlet integral[25] one finds

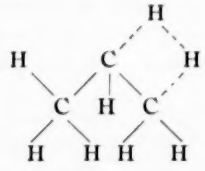
$$k = \frac{1}{h}\int_0^{E-\epsilon_0} \frac{(E-\epsilon_0-\epsilon_t)^{N-2}(N-1)!\prod_{i=1}^{N-1}\left(\frac{1}{h\nu_i^{\ddagger}}\right)}{(N-2)!E^{N-1}\prod_{j=1}^{N}\left(\frac{1}{h\nu_j}\right)}\,d\epsilon_t$$

$$k = \left(\frac{E-\epsilon_0}{E}\right)^{N-1}\frac{\prod_{j=1}^{N}\nu_j}{\prod_{i=1}^{N-1}\nu_i^{\ddagger}} \qquad (4)$$

An equation for the rate constant can be derived in a similar manner for the case where the reactant is taken to be a system of $N - L$ weakly coupled harmonic oscillators plus $L$ internal rigid rotors, and the activated complex a system of $N - L^{\ddagger} - 1$ harmonic oscillators plus $L^{\ddagger}$ internal rotors. The equation is

$$k = 2^{(L^{\ddagger}-L)/2}\pi^{L^{\ddagger}-L}\frac{\Gamma[N-(L/2)]\prod_{f=1}^{L^{\ddagger}} I_f^{\ddagger 1/2}\prod_{j=L+1}^{N}\nu_j}{\Gamma[N-(L^{\ddagger}/2)]\prod_{i=1}^{L} I_i^{1/2}\prod_{g=L^{\ddagger}+1}^{N-1}\nu_g^{\ddagger}} \times \left(\frac{E-\epsilon_0}{E}\right)^{N-(L/2)-1}(E-\epsilon_0)^{(L-L^{\ddagger})/2} \qquad (5)$$

Equation (5) is used in cases such as the following: For internal excitation high enough to cause decomposition of propane ions, it is very probable that the two methyl groups rotate freely about their C–C bonds. Appearance potential measurements indicate that the formation of $C_3H_6^+$ ion is accompanied by the formation of a hydrogen molecule, rather than two hydrogen atoms.[26] If one assumes the two lost hydrogens to have come from adjacent carbon atoms rather than the same one, then it is probable that in the activated complex the free rotation of one methyl group will have changed to a torsional vibration because of the formation of a weak H---H bond, as shown below.



The discussion so far has been for a single reaction only. If there are several competing reactions the theory should be equally applicable. One need only make the assumption that the rate of interchange of energy among the degrees of freedom of the reactant is sufficiently rapid so that the

formation of activated complexes for one reaction does not appreciably affect the rate of formation of activated complexes for other reactions.

Using the considerations outlined above, we have made calculations in an attempt to reproduce the mass spectrum of the propane molecule. A simple computation shows that there is a time interval of roughly $10^{-5}$ seconds between formation of an ion in the electron beam and the acceleration of the ion in the ion gun of the mass spectrometer. Ions formed during this time interval by unimolecular decomposition of the parent propane ions will be detected at their expected place on the mass scale. Any decomposition occurring later will, in general, result in loss of the ions from the collected ion current, due to the ion-optical characteristics of the spectrometer. The one exception to this rule, metastable ions, will be discussed later.

Using the following breakdown scheme for the principal ions observed in the propane mass spectrum,

$$\begin{aligned} C_3H_8^+ &\rightarrow C_3H_7^+ \rightarrow (C_3H_5^+ + C_3H_3^+ + C_3H^+) \\ &\rightarrow (C_3H_6^+ + C_3H_4 + C_3H_2^+) \\ &\rightarrow C_2H_5^+ \rightarrow C_2H_3^+ \\ &\rightarrow C_2H_4^+ \rightarrow C_2H_2^+ \end{aligned}$$

the relative amount of unimolecular decomposition along various competing reaction paths for a time $t = 10^{-5}$ seconds has been calculated as a function of the internal excitation energy of the parent ion.

The activation energies were calculated from appearance potentials, ionization potentials and thermal data.[8] The frequencies and moments of inertia were estimated from data on their neutral counterparts (propane ion data from molecule data, for example) and the same values used for the activated complexes derived from them, except where obvious changes were indicated. The latter has already been illustrated for the activated complex for the reaction

$$C_3H_8^+ \rightarrow C_3H_8^{+\ddagger} \rightarrow C_3H_6^+ + H_2,$$

where a methyl group free rotation in the excited ion was assumed to have become a torsional vibration in the activated complex. A further example is the reaction

$$C_3H_8^+ \rightarrow (C_2H_5\text{—}CH_2)^{+\ddagger} \rightarrow C_2H_5^+ + CH_3.$$

Here the activated complex is essentially a propane ion with one C–C bond stretched to several times the equilibrium separation. One may reasonably assume that this bond stretching results primarily in a decrease of the rocking frequencies of the loose methyl group in the activated complex.

The results of this calculation are shown in the breakdown graphs of

figures 1 and 2. These graphs show the relative amounts of fragment ions formed from a parent ion with given excitation energy at the end of $10^{-5}$ seconds. The two graphs were calculated and plotted for two different possible activated complexes associated with the formation of the propyl ion. The other activated complexes were the same.

At this point the arbitrary nature of the calculation is surely apparent. One is dealing with many more parameters than unknowns. However, this much can be stated: In so far as vibrational frequencies and moments of inertia are concerned, their function as arbitrary parameters is very much restricted by criteria of self-consistency among the various activated com-

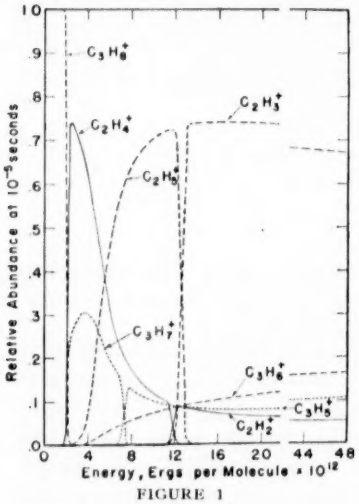



FIGURE 1

Fraction of the several fragment ions formed from propane ions in $10^{-5}$ seconds as a function of internal excitation energy.

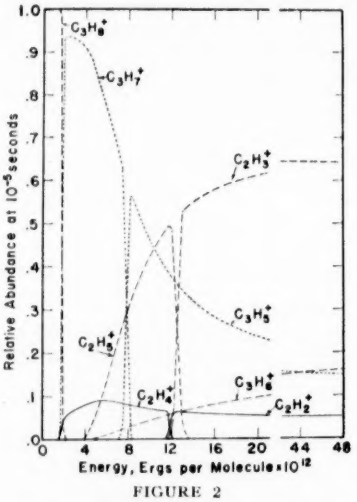



FIGURE 2

Same as figure 1, but calculated assuming a different activated complex for the formation of the propyl ion.

plexes and also by what might be termed consistent analogy to the values experimentally obtained on the corresponding neutral molecules.

The energies of activation are essentially experimental quantities, differences in appearance potentials. Their validity in use as activation energies hinges on the validity of the view-point which considers the mass spectrum of a large molecule in terms of a rate theory such as given above. We have been able to deduce breakdown schemes for both normal butane and isobutane and have calculated energies of activation which, in so far as they parallel the breakdown scheme for propane, are in good agreement:[27]

As previously discussed, theory has not yet provided a distribution func-

tion for internal excitation for polyatomic molecules. The probability of obtaining ions with no internal excitation is essentially zero because the minima on the potential surfaces of the molecule and the molecule-ion will, in general, have different equilibrium internuclear distances. The amount of fragmentation observed in mass spectra, "stopping power" measurements for high energy particles[28] and the relatively small amount of double ionization in mass spectra[29] all indicate that the mean of this distribution function is in the range of five to ten electron volts and that the function drops off quite rapidly for much higher energies. We have tried the following extremely simple distribution functions: (i) Unit probability in the range 0–20 $\times$ $10^{-12}$ ergs/molecule; zero probability above 20 $\times$ $10^{-12}$. (ii) Unit probability in the range 0–16 $\times$ $10^{-12}$; 0.5 probability in the range 16–20 $\times$ $10^{-12}$; zero probability above 20 $\times$ $10^{-12}$. (iii) Probability 0.9 in the range 0–4 $\times$ $10^{-12}$ ergs/molecule; 1.0 in the range 4–14 $\times$ $10^{-12}$; 0.5 in the range 14–20 $\times$ $10^{-12}$; 0.0 above 20 $\times$ $10^{-12}$. (1 $\times$ $10^{-12}$ ergs/molecule = 0.624 electron volt.)

With these distribution functions and the results shown in figure 1 the following mass spectra are obtained by graphical integration.

| | MASS SPECTRUM | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| DISTRIBUTION FUNCTION | $C_3H_8^+$ | $C_2H_7^+$ | $C_3H_6^+$ $C_3H_5^+$ $C_3H^+$ | $C_3H_6^+$ $C_3H_4^+$ $C_3H_2^+$ | $C_2H_6^+$ | $C_2H_4^+$ | $C_2H_3^+$ | $C_2H_2^+$ |
| (i) | 0.102 | 0.064 | 0.059 | 0.062 | 0.249 | 0.161 | 0.272 | 0.031 |
| (ii) | 0.114 | 0.071 | 0.056 | 0.057 | 0.277 | 0.178 | 0.220 | 0.027 |
| (iii) | 0.111 | 0.074 | 0.056 | 0.056 | 0.300 | 0.185 | 0.194 | 0.024 |
| Experimental | 0.090 | 0.071 | 0.103 | 0.042 | 0.310 | 0.183 | 0.122 | 0.027 |

Total ionization normalized to unity. Experimental peaks omitted total 0.052.

The agreement between the experimental values and those calculated for distribution function (iii) is remarkably good, inasmuch as with but one exception the parameters first estimated for the activated complexes were used in the calculation. Under these circumstances, the agreement is somewhat fortuitous but still indicative of the validity of the theory.

There is some evidence from studies with isotopically labeled molecules[30] that the kinetics summarized in figure 2 are preferable to those shown in figure 1. Similar calculations based on the results of figure 2 do not yield nearly as good agreement with experiment but do indicate that suitable adjustment of parameters would permit agreement to be obtained.

To eliminate the arbitrary features of this theory more data are required. Most useful would be the complete mass spectra for propane and the butanes for several electron voltages in the range 15 to 40 volts. The maximum excitation energy available for the parent ions is the electron energy minus the ionization energy of the molecule. The above voltage range would give a variation in the high-energy cut-off of the distribution function between 6 and 50 $\times$ $10^{-12}$ ergs/molecule and corresponding variations in

the mean value of the excitation energy. It is hoped that such data will be available shortly and that the validity of this theory can then be definitely demonstrated.

Several others aspects of mass spectra are explainable in terms of this theory, namely, temperature dependence, metastable transitions and isotope effects.

The essential feature of temperature effects in mass spectra is the marked decrease of parent ion intensity with increasing temperature.[31] Fragment ion intensity changes are much smaller and may have either sign. In terms of this theory, increased temperature shifts the energy distribution function maximum to slightly higher values and causes it to rise less steeply from its near-zero value at zero energy. As may be seen from figures 1 or 2, this results in a smaller relative abundance of the parent ion in the calculated mass spectrum. Changes in intensities of other mass peaks will depend upon how the change in the energy distribution function affects the integrals of the product of the distribution function with the relative breakdown curves such as are given in figures 1 and 2.

Careful scanning of the mass spectrum of many molecules at non-integral mass values has revealed the presence of numerous diffuse peaks.[32] These peaks result from decompositions of ions into smaller ions and neutral fragments in the time interval occurring between the exit of the ions from the electrostatic accelerating field an1 their entry into the magnetic analyzing field. Their intensity is rarely greater than one per cent of maximum peak intensity in a typical mass spectrum. In terms of the proposed theory their significance is twofold. All metastable transitions involve decompositions which are similar to those transitions proposed here as the decomposition reactions by means of which the mass spectrum of a typical molecule is formed.[33] This is substantiation in the sense that the proposed decompositions, arrived at by consideration of the energies involved, do actually occur to at least some extent. Furthermore, the energy distribution function will yield a small fraction of excited ions having an amount of internal excitation which will give rise to decompositions with a half-life centered about the value $10^{-6}$ seconds, the experimental value measured by Hipple.[34] The theory presented here implies that metastable ions of all half-lives should exist. The experimental value quoted above was actually determined by the geometry and fields of the mass spectrometer used and does not indicate that metastable ions of other half-lives are absent. Quantitative calculations of metastable peak heights and comparison with experiment will also be undertaken as soon as suitable data are available.

As can readily be seen from the rate equation derived above, a detailed discussion of isotope effects would require a much better knowledge of physical parameters such as frequencies, moments of inertia and activation

energies than is presently available. However, one interesting series of experiments can be quite simply discussed. Introducing a deuterium atom into a hydrocarbon speeds up the dissociation of any one of the remaining hydrogens. However, this deuterium comes off less readily than did the hydrogen it replaced.[30]

The expression in the rate equation for the density of states of a collection of harmonic oscillators does not take the zero-point energy of the oscillators into account, since the expression is a derivative. Following Marcus,[23] we call the internal vibrational energy minus the zero-point energy the "non-fixed" energy. It is clear then that the internal energy $E$ in the rate equation refers to this non-fixed energy rather than to the total vibrational energy. In terms of this quantity then, the replacement of other hydrogens by deuterium atoms results in increased non-fixed energy. Hence the rate of decomposition will increase if all other conditions are kept the same; in other words a hydrogen atom will now come off more easily. However, substitution of the given hydrogen atom by a deuterium atom results in a slower decomposition rate (or probability) because of a decrease in the "frequency factor" of the rate equation and an increase in the activation energy necessary to break the C–D bond.[35]

*Summary.*—The mass spectra of polyatomic molecules have been discussed in terms of a series of quasi-equilibrium unimolecular decompositions. An absolute rate theory for isolated systems having a large but finite number of degrees of freedom has been presented. The application of the rate theory to such a decomposition scheme for propane gives results in agreement with experiment. Due to the large number of parameters and approximations this agreement is not conclusive indication of the validity of the theory. Additional qualitative supporting evidence is also presented. The theory will serve as a basis for further calculations and experiment.

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† U. S. Atomic Energy Commission Predoctoral Fellow, 1950–1952.

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# THE STRUCTURE OF STABLE PENTABORANE

BY KENNETH HEDBERG, MORTON E. JONES AND VERNER SCHOMAKER

GATES AND CRELLIN LABORATORIES OF CHEMISTRY,* CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

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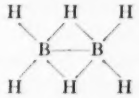
Stable pentaborane, $B_5H_9$, is one of the boron hydrides, a short, comparatively little studied series of extraordinary compounds for which a satisfactory elementary valence theory is lacking. In 1947 we decided to undertake new electron diffraction studies of the molecular structures. The early diffraction work and most of the theoretical discussion had been too much influenced (it now seems) by unfortunate analogies to ordinary valence compounds, and it had become reasonably clear that at least the old, ethane-like structure for diborane was incorrect and that a bridge structure (I) was more likely. In the case of $B_5H_9$, also, the structure from



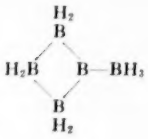

(I)

the previous diffraction study[1] was not in complete agreement with the appearance of the photographs, one of which was available to us. The bridge structure of diborane has now been well established,[2] the crystal structure of decaborane ($B_{10}H_{14}$) has been determined,[3] and the $B_5H_9$ structure has been determined, both from the gas diffraction pattern in the work here to be described[4] and from an x-ray study of the crystal by Dulmage and Lipscomb.[5] The most impressive attempt at a theory of the compositions and structures, however—Pitzer's protonated double bond theory,[6] which based the structures of all the boron hydrides on diborane bridges and on some plausibly assumed conjugation properties of these bridges—has been a casualty: each of the new boron hydride structures has shown little over-all relation to the previous ones and neither involves the diborane bridge.

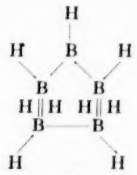
*The Structure Determination.*—The method used has been outlined in recent reports from this laboratory.[7]

New photographs were taken with samples kindly provided by Professor H. I. Schlesinger of the University of Chicago and by Doctor I. Shapiro of the Naval Ordnance Test Station, Pasadena. The camera distance was 10.94 cm. and the electron wave-length 0.0608 Å. Independent visual interpretations of the photographs were made by two observers (see Fig. 1).

The radial distribution curves, showing only two strong peaks, at 1.74 Å. (B—B) and 2.57 Å. (B···B and B···H), exclude both the structure advocated in the original study[1] (II) and that proposed by Pitzer[6] (III):



(II)



(III)

II would requre significant B···B interactions at $1.74\sqrt{2} = 2.46$ Å. and at $2 \times 1.74 \sin 135°/2 = 3.22$ Å., at least if it were normally rigid, and III at an average of $2 \times 1.74 \sin 108°/2 = 2.82$ Å. (The original specification of III would also require the 1.74 Å. peak to be obviously doubled.) The radial distribution curves did not lead directly to the structure, mainly because neither the relative areas of the widely separated main peaks nor the indicated absence of minor interactions outside them could be relied upon.

Nevertheless, the radial distribution information provided a starting point for a more detailed analysis of the visual curves themselves. This analysis first showed that the observed doubled character of maximum 9–10 requires two groups of B—B interactions, of about equal weight, separated by 0.11 ± 0.01 Å. Even then, the outer part of the observed intensity curve, including max. 9–10, could not be reproduced without severely restricting the distribution of weights and distances within the 2.57 Å. radial distribution peak, either by making the distribution essentially continuous (corresponding to severe "temperature" factors) or in other ways which, given the B—B split, were fairly obvious. Finally, when this was done on the assumption that the 2.57 Å. peak was due mainly to rigid B···B interactions, it appeared that the B—H terms were probably also split, by about 0.15 Å. into two groups of about equal weight. Corresponding to this distance information three unsymmetrical arrangements of the boron atoms, a puckered five-membered ring, a dimethylcyclopropane-like arrangement, and an ethylcyclopropane-like arrangement, all actually rather closely similar, were found.

Before constructing and testing actual models based on these arrangements of boron atoms (the theoretical intensity curves already calculated lacked the B···H terms), we decided to re-examine the tetragonal pyramid arrangement, which had been considered but rejected in the original diffraction study, had more recently been further advocated by Pauling,[8] and, unlike our unsymmetrical arrangements, was in agreement with recent indications of high symmetry from spectroscopic[9] and calorimetric[10] data. The 2.57 Å. peak now had to be attributed mainly to B···H rather than B···B interactions, contrary to our previous assumption,[11] but with the help of the previous analysis a suitable disposition of hydrogen atoms was readily found (Fig. 2) and all others of full symmetry ($C_{4v}$) were ten-

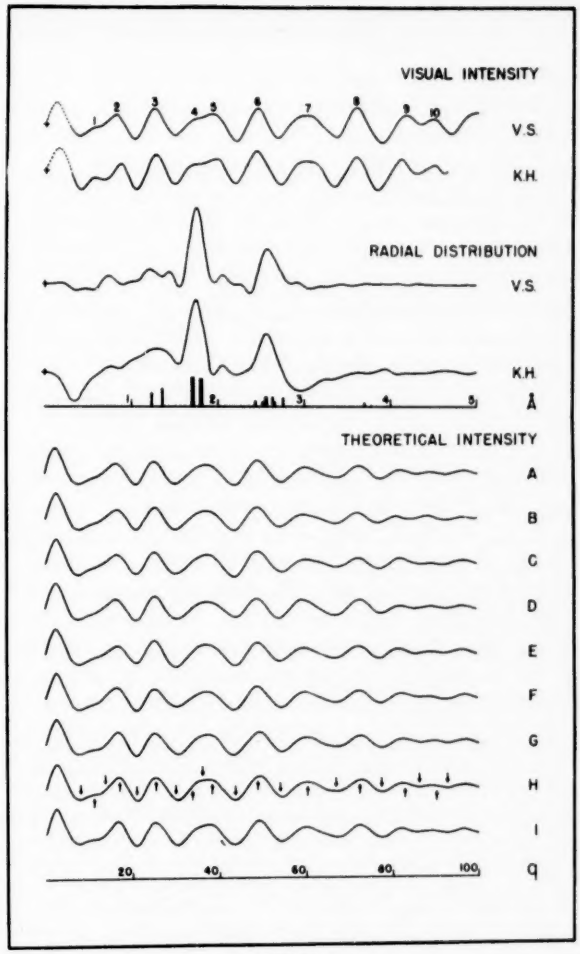



FIGURE 1.

Electron diffraction curves. The theoretical intensity curves are for the following pyramidal models:

| Curves | B—$H_{av.}$/B—$B_{av.}$ | B—B split | B—H split | $<B_1B_2B_2$ | external dihedra. $<B_1B_2B_3$-$B_2B_2H_4$ |
|---|---|---|---|---|---|
| *A* | 1.275/1.740 | 0.100 | 0.150 | 90° | 180° |
| *B* | 1.275/1.745 | 0.110 | 0.150 | 90° | 180° |
| *C* | 1.275/1.740 | 0.100 | 0.050 | 90° | 180° |
| *D* | 1.275/1.740 | 0.100 | 0.250 | 90° | 180° |
| *E* | 1.225/1.740 | 0.100 | 0.150 | 90° | 180° |
| *F* | 1.325/1.740 | 0.100 | 0.150 | 90° | 180° |
| *G* | 1.275/1.740 | 0.100 | 0.150 | 115° | 180° |
| *H* | 1.275/1.740 | 0.100 | 0.150 | 115° | 190° |
| *I* | 1.275/1.740 | 0.100 | 0.150 | 125° | 190° |

tatively eliminated. The new structure, with the $B_2$—$B_2$ and $B_2$—$H_3$ distances the longer of their respective kinds, seemed plausible and met with immediate success.

All except H · · · H terms were included for the theoretical intensity curves. The coefficients $a_{ij}$ of the temperature factors $\exp(-a_{ij}q^2)$ were taken as 0.00016 for $B_2$—$H_2$ and $B_1$—$H_1$, 0.00028 for $B_2$—$H_3$, 0.00060 for B · · · H, and zero otherwise, as for diborane,[2b] and the effective value 1.25 was used for $Z_H$. Of the selection of curves shown in Fig. 1, *G*, *H*, and *I* are acceptable, *A*, *B*, and *C* are doubtful, and *D*, *E*, and *F* are unacceptable. Important items for these conclusions are the depth of min. 4, the shape of doublet 4-5, the relative intensities of minima 6, 7, 8, and 9, the shape of max. 7-min. 8, and the position and shape of doublet 9-10. For the best curves, the only point of substantial disagreement concerns the heights of the first three main maxima; it is almost inconsequential for the parameter determination and probably arises from an underestimate, such as could be expected, of the height of the broad inner max. 1-2.

In terms of $B—B_{av.} = 1.740$ Å. the best shape parameter values and estimated limits of error, together with the ranges for which intensity curves were calculated, are: $B—H_{av.}$, 1.288 ± 0.044 Å. (1.22–1.35 Å.); $B—H_{split}$, 0.125 ± 0.090 Å. (0.05–0.35 Å.); $B—B_{split}$, 0.105 ± 0.010 Å. (0.09–0.12 Å.); ∠ $B_1—B_2—H_2$, 120 ± 20° (85–125°); and external dihedral angle $B_1B_2B_2$-$B_2B_2H_3$, 187 ± 10° (165–200°), all for the assumed $C_{4v}$ symmetry. These values and the values of $\langle q_{calc.}/q_{obs.}\rangle_{av.}$ (see table 1 for an example) lead to the following results for the bond lengths: $B_1—B_2$, 1.700 ± 0.017 Å.; $B_2—B_2$, 1.805 ± 0.014 Å.; $B_1—H_1$ and $B_2—H_2$, 1.234 ± 0.066 Å. ($B_1—H_1 = B_2—H_2$ assumed); and $B_2—H_3$, 1.359 ± 0.077 Å.

The limits of error are conservative except that no allowance has been made for the possible effects on the angle determinations of our rough assumption that the previous guess for $a_{B\cdots H}$ in diborane should apply to $B_5H_9$, for all the different B · · · H terms. The concentration of all the B · · · H distances within the 2.57 Å. peak makes the question of interaction between temperature factor and distance parameters more serious than usual, but the boron parameters and probably the B—H distances should not be much affected, since they are determined largely by the outer part of the pattern, where the B · · · H contribution is in any case small. It may be noted that the crystal[5] and gas values for the bond angles and bond lengths in $B_5H_9$ are in good agreement except for the B—B lengths, for which the crystal values (1.66 ± 0.02 Å. and 1.77 ± 0.02 Å.) are shorter than ours by possibly significant amounts compared to the limits of error. Our B—B lengths, however, are in good agreement with the preliminary results 1.69 Å. and 1.80 Å. of a recent microwave investigation,[12] from which none of the other parameter values have yet been reported.

The x-ray confirmation of the structure type, which was communicated to us during our parameter determination, made unnecessary any further study of other possibilities, including the unsymmetrical ones described above. The high over-all symmetry and especially the $C_{4v}$ skeletal symmetry have also been confirmed by the microwave investigation.[12]

*Discussion.*—The $B_5H_9$ structure has high ligancies, two for the bridge hydrogen atoms, five for the apical boron atom, and six for the basal boron

atoms, in agreement with the principle[13] that electron deficiency gives rise to structures showing ligancies in excess of the respective numbers of suitable atomic orbitals. For the count of ligands, we take the direct B—B interaction of a bridge bond as bonding, although the related B···B interaction in diborane is often regarded as not bonding. We believe our assumption is the more likely one in view of the comparatively short B—B distance. It also makes the ligancies of hydrogen and boron in the boron hydrides and other high-ligancy compounds of boron more uniformly consistent with the high-ligancy principle, and is the natural assumption to make if these compounds are to be related to ordinary covalent compounds in terms of resonance, following Pauling's discussion of the metals.[14] Pauling's relation $r_n = r_1 - 0.300 \log_{10} n$ yields an attractive correlation of the actually very widely varying bond distances,[15] as well as inferences about certain other aspects of the structures. For example, the boron radius which would be exactly compatible with the bond distances of our preliminary report for $B_5H_9$ is 0.795 Å., in excellent agreement with the average 0.794 Å. obtained from all these compounds.

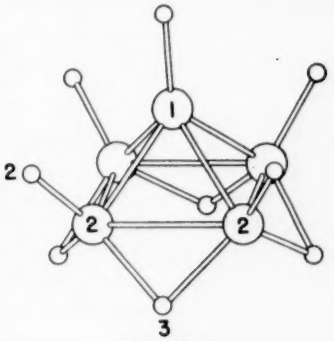


FIGURE 2.
The $B_5H_9$ structure.

The basal boron atoms of $B_5H_9$ and the apical boron atoms of $B_{10}H_{14}$ form just the same set of bonds; similarly, the apical boron atoms of $B_5H_9$ resemble the boron atoms of the calcium boride structure in an octahedron arrangement except for replacement of external B by H. As King and Lipscomb pointed out,[16] moreover, the whole $B_5H_9$ structure is related to the calcium boride structure in almost precisely the same way as the $B_{10}H_{14}$ structure is related to the boron carbide structure.[17] We may add that the basal boron atoms of $B_5H_9$ and all the boron atoms of decaborane, as well as the boron atoms of boron carbide and the icosahedron atoms of elementary boron (in the modification of known structure[18]), all have six ligands in the icosahedron arrangement, with bond angles approximating the ideal values of 60°, 108°, and $121^3/_4°$ about as well as would seem possible under the constraints imposed by differing bond lengths and incompatible over-all symmetries.[19] Accordingly, it seems reasonable to suggest that these structures all reflect a strong tendency for sexiligated boron to adopt approximately the ideal icosahedron arrangement.

The occurrence of the icosahedron and octahedron arrangements is remarkable because they are notably anisotropic, in violation of what might be expected to result from the $sp^2$ (and $sp^3$, for the hydrides) hybrid

orbitals on which the bonding is presumably mainly based and because there is surely no lack of more conventional alternatives. For elementary boron, for example, ordinary octahedral coordination in the simple cubic structure would seem suitable, especially in view of its frequent occurrence in complex structures for other atoms which are regarded as forming six half-bonds.[20] To be sure, the icosahedron and octahedron arrangements would seem less anisotropic if the external bonds were stronger than the internal bonds, as indeed is the general indication for $B_5H_9$ and decaborane. For the basal boron atoms of $B_5H_9$, for example, the bridge B—H, bridge B—B, and slant B—B bonds have the respective Pauling bond numbers 0.46, 0.45, and 0.67, with a total of 2.49, or only about three times the bond number 0.77 of the B—H external bond.[15] But for boron carbide, ele-

TABLE 1

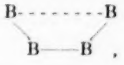
COMPARISONS OF OBSERVED AND CALCULATED POSITIONS OF MAXIMA AND MINIMA FOR MODEL H

| | $q_{obs.}$ | | | | $q_{calc.}/q_{obs.}$ | | | |
|---|---|---|---|---|---|---|---|---|
| | V. S. | | K. H. | | V. S. | | K. H. | |
| NO. | MAX. | MIN. | MAX. | MIN. | MAX. | MIN. | MAX. | MIN. |
| 1 | 11.19 | 7.76 | 10.89 | 8.06 | (0.956) | (0.979) | (0.983) | (0.943) |
| 2 | 16.41 | 13.31 | 17.31 | 13.88 | (1.012) | (0.939) | (0.959) | (0.901) |
| 3 | 25.00 | 20.47 | 25.34 | 21.03 | 1.012 | 1.021 | 0.998 | 0.994 |
| 4 | 33.87 | 29.70 | 33.33 | 29.77 | (1.025) | 1.024 | (1.041) | 1.021 |
| 5 | 38.52 | 35.41 | 39.66 | 36.42 | (0.971) | (1.011) | (0.943) | (0.983) |
| 6 | 48.70 | 43.46 | 48.59 | 43.62 | 1.008 | 0.999 | 1.011 | 0.995 |
| 7 | 59.51 | 53.53 | 60.45 | 54.25 | 1.000 | 1.014 | 0.991 | 1.007 |
| 8 | 72.66 | 66.95 | 71.87 | 66.61 | 0.998 | 1.004 | 1.009 | 1.009 |
| 9 | 83.29 | 78.03 | 82.23 | 76.50 | (0.984) | 0.993 | (0.997) | 1.013 |
| 10 | 90.07 | 86.50 | 89.97 | 85.57 | (0.989) | 0.991 | (0.991) | 1.002 |
| 11 | .. | 93.23 | .. | 91.89 | ... | 0.992 | ... | 1.007 |
| | | | Average, 12 features | | 1.0047 | | 1.0048 | |
| | | | Average deviation | | 0.009 | | 0.007 | |

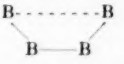
mentary boron, and calcium boride there is no definite indication one way or the other. Altogether, a proper understanding of the details of the bonding is lacking.

Nevertheless, it seems unlikely that the immediate bond arrangement in these structures is superior. Instead, the essential point may be that they allow an increase in ligancy without a corresponding increase (or even with a decrease) in the number and severity of close non-bond interactions: compare, for example, the joined icosahedron unit of the boron and boron carbide structures with the simple cubic structure. In the latter, each atom has twelve next-nearest neighbors related to it by 90° bond angles, whereas the icosahedron atom has only five internal next-nearest neighbors at 108° and five external next-nearest neighbors at 122°. This strongly suggests that the next-nearest interactions are repulsive and important

and that the high-ligancy principle should be revised to say that the high ligancies tend to be achieved in such a way as to minimize the numbers and maximize the distances of next-nearest neighbors, even if the resulting bond arrangements would appear by standards of ordinary covalence to be unduly strained. It may also account for the lack of apparent extra strength of the external bonds where two octahedra or two icosahedra are joined: for the icosahedron, again, each external bond would be opposed principally by ten next-nearest interactions at 122° and ten second-nearest interactions (assuming the staggered orientation of groups about the external bond) of the type



whereas each internal bond is opposed (a full counting shows) by only one internal next-nearest interaction at 108°, two external next-nearest interactions at 122°, and one external interaction of the type



in the opposed orientation. The present situation is evidently related to the cases of cyclopropane and cyclobutane,[21] where the energy and C—C bond length in cyclobutane are both greater than normal, apparently because of cross-ring repulsion, while in cyclopropane, in which the repulsion is avoided by formation of the three-membered ring, the bond length is less than normal and the energy still greater than normal, both apparently in consequence of the angle strain. In the high-ligancy boron compounds the relationships are no doubt different, especially because of the complicated resonance situation; nevertheless, the importance of next-nearest neighbor repulsions seems to be verified and there is the additional indication that angle-strain shortening of the internal bonds may also occur. For the calcium boride structure, of course, the role of the metal atoms has also to be considered.

We should like to express our thanks to Professor Pauling for his continued helpful interest in the investigation.

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[8] Private communication.

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[11] We had at first rejected the tetragonal pyramid (see reference 4), mainly because its relative number of B · · · B interactions and relative B · · · B distance ($1.74\sqrt{2} = 2.46$ Å.) are in disagreement with this false interpretation of the 2.57 Å. peak and because, in view of this interpretation, little attention was given to the (crucial) disposition of the hydrogen atoms. Our initial failure to recognize the B—B split also contributed to the argument on the apparent B—B—B angle (i.e., the ratio 2.57/1.74). Actually, the half-width of the 1.74 Å. peak is somewhat greater than would be expected from our convergence factor for zero split (although less than for a 0.10 Å. split). We overlooked this, failing to consider how little widening would result from distance splits up to 0.10 Å., especially in the absence of a well-resolved, sharp term to make the slow decline of the split-term contribution obvious in the appearance of the photographs. (In reference 13 the radial distribution distances were misquoted as 1.76 Å. and 2.62 Å.; also, in the discussion of the puckered five-membered ring model, it was not made clear that for the theoretical curve mentioned the hydrogen terms were neither based on an actual model nor complete. The complete curve was no longer promising; the original curve, lacking the B—B split, of course never was a good fit to the outer part of the pattern.)

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[19] Our values for $B_3H_9$ are $\angle B_1B_2B_2 = 58°$, $\angle B_2B_2H_3 = 48\frac{1}{2}°$, and $\angle H_3B_2H_3 = 113°$; $\angle B_1B_2H_3 = 106°$, $\angle B_2B_2H_3 = 113°$, and $B_2B_2B_2 = 90°$; and $\angle H_2B_2B_1 = 120°$, $\angle H_2B_2B_2 = 134°$, and $\angle H_2B_2H_3 = 108°$. Here the four-membered ring, the differences of B—H (bridge) and B—B bond lengths, and the evident need for large $\angle H_3B_2H_3$ to provide a reasonably long $H_3 \cdots H_3$ distance, which we presume to be non-bonding, are obviously serious constraints; nevertheless, the average external angle is 121°, in surprising agreement with the ideal value. Also, our value 120° for $\angle B_1B_2H_2$ approximately equalizes the external angles, and the smaller, crystal value of 115°, which by the quoted ±5° limit of error is considerably more reliable than ours, equalizes the apparent strains very well indeed.

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# AN INDUCED MATERNAL EFFECT ON THE STABILITY OF THE RING-X-CHROMOSOME OF DROSOPHILA MELANOGASTER

BY SPENCER W. BROWN AND ALOHA HANNAH

GENETICS DIVISION AND ZOOLOGY DEPARTMENT, UNIVERSITY OF CALIFORNIA, BERKELEY

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The ring-X-chromosome of *Drosophila melanogaster* has been recognized as a potentially unstable chromosome since the work of L. V. Morgan.[1, 2] This instability is expressed by loss of the ring-X, resulting in gynandromorphism and the expression of recessive, sex-linked factors in appropriate tissues of certain heterozygotes. Although the percentage of gynandromorphs obtained by the use of the $X^{c2}$-ring is usually low (0.23–0.68%, Battacharya[3]; Braver and Blount[4]), mutational changes induced in the ring chromosome have raised the incidence appreciably (Braver and Blount[4]; Griffen and Lindsley[5]). It will be demonstrated in the present report that environmental changes can also influence the stability of the $X^{c2}$-chromosome.

During the course of an experiment set up for other purposes, it became apparent that the introduction of a ring-X-chromosome into eggs produced by aged virgins resulted in a marked increase in the incidence of gynandromorphs formed as a result of an elimination of the ring chromosome. Male flies with the X-chromosome factors, *y w spl* $sn^2$, were irradiated and mated with *Muller-5*-females. The virgin $F_1$ females were then mated with $X^{c2}$-males and the *y w spl* $sn^2/X^{c2}$-class of female offspring was examined for evidence of gynandromorphism. The $F_1$ flies which had been mated within two days after emergence gave significantly fewer gynandromorphs than those which had been aged 7 days or longer before mating.

The effect of aging was shown to produce similar results when a second trial was made with unirradiated chromosomes in the same sequence of crosses. In this experiment, as a further control, unaged $F_1$ virgins were mated at the time aged $F_1$ virgins were originally collected, and again when the aged $F_1$ virgins were mated. The results obtained with the unirradiated individuals are essentially similar to those from the irradiated flies; the data from these two series have therefore been combined. Among 4183 *y w spl* $sn^2/X^{c2}$-flies from $F_1$ females aged 0–2 days, 20 gynandromorphs were found while 233 gynandromorphs were observed in 5643 flies from females aged 8–17 days. $\chi^2$ for these figures is 126.18, and $p < 0.01$ Within single trials, the aged females always produced more gynandromorphs than the unaged, and the ranges for these two classes did not overlap, running from 0–1.6% for the unaged and 2.0–8.0% for the aged. In each series, heterogeneity was statistically significant but was not assignable to different duration of aging within the periods cited above. The oc-

currence of such unexplained heterogeneity is a fact of importance in the consideration of the material to follow.

With the same sequence of crosses again being used, comparisons were made among several genotypes as to the incidence of gynandromorphs in offspring of the first 24-hr. laying period of $F_1$ females (table 1). Relatively great differences in percentage of gynandromorphs are to be noted among the various genotypes. Because of similar fluctuations among repeated trials of the same cross, possible independent influence of genotype cannot at present be properly evaluated. Also the influence of the *Muller-5*-chromosome, used in all experiments reported here, has yet to be determined.

TABLE 1

INCIDENCE OF GYNANDROMORPHS FROM AGED AND UNAGED $F_1$ VIRGINS OF VARIOUS GENOTYPES, IN FIRST 24-HR. LAYING PERIOD AFTER MATING

| GENOTYPE OF $F_1$ ♀ ♀ PARENT | DAYS AGED | *X-ple/Xc²* ♀ ♀ | GYN | % | *M-5/Xc²* ♀ ♀ | GYN | % |
|---|---|---|---|---|---|---|---|
| *M-5/ y w spl sn²* | 0–1 | 776 | 3 | 0.39 | 617 | 1 | 0.16 |
| *M-5/y w spl sn²* | 9–15 | 968 | 29 | 7.13 | 890 | 47 | 5.28 |
| | | $\chi^2 = 44.32^d$ | | | $\chi^2 = 28.22^d$ | | |
| *M-5/y sc m f⁵* | 0 | 372 | 3 | 0.80 | 290 | 4 | 1.02 |
| *M-5/y sc m f⁵* | 11 | 91 | 32 | 26.02 | 105 | 20 | 16.00 |
| | | $\chi^2 = 85.55^d$ | | | $\chi^2 = 31.84^d$ | | |
| *M-5/y ac sn³* [e] | 0 | 392 | 3 | 0.77 | 320 | 1 | 0.66 |
| *M-5/y ac sn³* [e] | 11 | 2033 | 171 | 7.76 | 1854 | 77 | 3.91 |
| | | $\chi^2 = 25.25^d$ | | | $\chi^2 = 10.03^d$ | | |
| *y ac sn³/M-5* [e] | 11 | 720 | 29 | 3.87 | 689 | 16 | 2.27 |
| *M-5/dl-49, y Hw v⁶m² f* | 11 | 617 | 58 | 8.59 | 608 | 39 | 6.03 |
| *M-5/y ac sc⁸ w^a* [e] | 11 | 479 | 6 | 1.24 | 421 | 10 | 2.32 |
| *y ac sc⁸ w^a/M-5* [e] | 11 | 508 | 12 | 2.31 | 436 | 5 | 1.13 |

[d] For all $\chi^2$, df = 1, p. $<0.01$.

[e] In reciprocal crosses, X of maternal parent cited first.

No gynandromorphs were found in similar crosses in which males with a rod-chromosome, wild-type or with genetic markers, were substituted for males with the $X^{c2}$-chromosome in matings with aged $F_1$ virgins. These data include 1591 females of the *y w spl sn²* or *y ac sn³*/rod- and 1089 of the *M-5*/rod-class.

On daily transference of the mated $F_1$ females to new cultures, the eggs produced during the first 24 or 48 hr. after mating yielded a higher percentage of gynandromorphs than those produced subsequently (table 2). This relationship held true also for the *M-5*/$X^{c2}$-class from the various crosses. Following the initial high incidence, the percentage of gynandromorphs approached that observed for the standard 8-day laying period. The rate of decline, however, was not uniform.

If the $F_1$ virgins were aged in bottles to which no fresh yeast had been added, their production of gynandromorphs was decreased in comparison

TABLE 2

GYNANDROMORPHS OBTAINED FROM SUCCESSIVE 24-HR. LAYING PERIODS AFTER MATING

| SERIES[a] | DAYS AGED | CLASS OF FLIES | 1ST ♀♀ | 1ST % GYN | 2ND ♀♀ | 2ND % GYN | 3RD ♀♀ | 3RD % GYN | 4TH ♀♀ | 4TH % GYN |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0–1 | *y w spl sn³/X c²* | 776 | 0.39 | 205 | 0.97 | .. | .. | .. | .. |
| | | *M-5/X c²* | 617 | 0.16 | 223 | 0.89 | .. | .. | .. | .. |
| 2 | 9–15 | *y w spl sn³/X c²* | 968 | 7.13 | 743 | 9.06 | 303 | 4.04 | 281 | 4.10 |
| | | *M-5/X c²* | 890 | 5.28 | 665 | 5.81 | 285 | 3.06 | 265 | 1.85 |
| 3 | 0 | *y ac sn³/X c²* | 392 | 0.77 | 333 | 0.90 | .. | .. | .. | .. |
| | | *M-5/X c²* | 320 | 0.31 | 359 | 0.00 | .. | .. | .. | .. |
| 4[b] | 11 | *y ac sn³/X c²* | 2033 | 7.76 | 1793 | 0.94 | 680 | 1.45 | .. | .. |
| | | *M-5/X c²* | 1854 | 3.91 | 1552 | 0.45 | 600 | 0.50 | .. | .. |
| 5[b] | 11 | *y ac sn³/X c²* | 720 | 3.87 | 742 | 1.46 | 443 | 0.67 | .. | .. |
| | | *M-5/X c²* | 689 | 2.27 | 584 | 1.18 | 394 | 1.01 | .. | .. |

[a] All series include data from females carried through one or two periods only.
[b] Series 4 and 5 are reciprocal parental crosses.

TABLE 3

THE EFFECT OF AGING $X^{c2}$-MALES ON THE INCIDENCE OF GYNANDROMORPHS IN THEIR PROGENY ON MATING TO $F_1$ VIRGIN FEMALES AGED 11 DAYS

| AGE OF PARENT ♂♂ IN DAYS | *y ac sn³/Xc²* ♀♀ | *y ac sn³/Xc²* GYN | *y ac sn³/Xc²* % | *M-5/Xc²* ♀♀ | *M-5/Xc²* GYN | *M-5/Xc²* % |
|---|---|---|---|---|---|---|
| 0.5 | 357 | 61 | 14.59 | 358 | 33 | 8.44 |
| 1–5 | 429 | 31 | 6.74 | 375 | 15 | 3.85 |
| 6+ | 471 | 20 | 4.07 | 403 | 8 | 1.95 |
| Homogeneity $\chi^2$ | | 34.04 | | | 19.75 | |
| df = 2, p< | | 0.01 | | | 0.01 | |

with that of females aged with yeast. $F_1$ *y ac sn*$^3$/*M-5*-virgins were aged 10–14 days. Of 1832 *y ac sn*$^3$/$X^{c2}$ flies produced by females aged without fresh yeast, 70 proved to be gynandromorphs while those aged with fresh yeast gave 196 gynandromorphs among a total of 2773. $\chi^2$ for these figures is 21.5, and $p < 0.01$.

The age of the males providing the $X^{c2}$-chromosome was another demonstrable factor influencing the incidence of gynandromorphism. In crosses with aged virgins, the younger males produced a significantly higher percentage of gynandromorphs (table 3).

There was no effect of an added Y-chromosome on the frequency of gynandromorphism; the percentage of gynandromorphs in XXY-cultures has not been significantly higher than that in XX-cultures.

TABLE 4

TYPES OF MOSAIC INDIVIDUALS PRODUCED BY AGED $F_1$ FEMALES OF VARIOUS GENOTYPES: (1) *M-5/y ac sn*$^3$,[a] (2) *M-5/y sc m f*$^5$, (3) *M-5/dl-49, y Hw v*$^0$ *m*$^2$ *f*

| | *X-ple*/$X^{c2}$ GYNANDROMORPHS | | | | |
|---|---|---|---|---|---|
| | (1) | (2) | (3) | Total | % |
| Elimination of (paternal) $X^{c2}$-chromosome | 526 | 49 | 56 | 631 | 96.8 |
| Number classified | 447 | 49 | 56 | 552 | |
| Approximate extent of male area: $<1/4$ | 231 | 29 | 30 | | |
| $1/4$ | 86 | 11 | 8 | | |
| $1/2$ | 82 | 3 | 7 | 262 | 47.5 |
| $3/4$ | 40 | 4 | 10 | 157 | 28.4 |
| $>3/4$ | 8 | 2 | 1 | | |
| Elimination of maternal chromosome | 7 | 0 | 1 | 8 | 1.2 |
| Elimination of maternal and paternal chromosomes | 0 | 1 | 0 | 1 | 0.2 |
| Somatic crossing-over | 1 + 1? | 0 | 0 | 2 | 0.3 |
| Somatic crossing-over and elimination | 6 | 1 | 1 | 8 | 1.2 |
| Special[b] | 2 | 0 | 0 | 2 | 0.3 |
| Total number of mosaics | 543 | 51 | 58 | 652 | |

[a] Reciprocal crosses combined.

[b] Unexpected eye color mosaics, not further analyzed.

Elimination of the $X^{c2}$-chromosome accounted for nearly all the observed mosaicism. Approximately bilateral gynandromorphs constituted 28% of this type of mosaic while almost 50% had one-fourth or more of the individual affected (table 4). It was impossible in these experiments to detect directly those flies from which the $X^{c2}$-chromosome might have been eliminated from all demonstrative tissue. That such complete eliminations might have occurred, however, was suggested by the fact that, in one test unaged $F_1$ females yielded 53.3% males among 1432 offspring while aged $F_1$ females gave 61.0% males among 636. Although both sex-ratios represent significant departures from the 1:1 expectancy ($\chi^2 = 6.04$ and 30.38, resp.), a comparison of the two ratios also reveals a highly signifi-

cant difference between the two ($\chi^2 = 10.33$). Secondly a number of M-5-males were observed which had phenotypic characteristics of the XO-constitution such as achaete and yellow patches of the thorax.

The arbitrary classification of gynandromorphs was limited to those individuals with patches of mosaic tissue large enough to be easily visible with 15× magnification. The single-bristle and other small spot mosaicism including singed or yellow as well as yellow-singed phenotypes, presented a different problem and will be mentioned again below.

In addition to the gynandromorphs attributable to the elimination of the $X^{c2}$-chromosome, a small percentage of other types of mosaic flies was observed. Data on the other mosaics are presented only for certain series of experiments (table 4). Similarly small percentages of the other types of mosaicism have, however, been observed in the other cultures throughout the period of study.

*Discussion.*—The above results demonstrate that three environmental factors can influence the stability of the $X^{c2}$-chromosome. These are (1) the age of the virgin female at mating, (2) the nutrition of the virgin female during aging, and (3) the age of the $X^{c2}$-male at mating. The first two factors affect a protoplasmic milieu into which the ring-X-chromosome is to be introduced, but from which it is absent during the period when these factors are producing their effects. If extent of mosaicism is an indication of time of elimination almost half of the observed gynandromorphs are the result of elimination at the first or second cleavage. On the basis of these observations, it may be concluded that the protoplasmic environment into which it is introduced may have a profound and immediate effect upon the stability of the $X^{c2}$-chromosome.

Elimination of the $X^{c2}$-chromosome might be attributable, *a priori*, either to an elimination independent of the other X-chromosome or to an elimination following somatic crossing over between the two X-chromosomes. The latter explanation will not account for the observed gynandromorphism for the following reason. Single crossing over in the four-strand state between a ring and a rod would lead to a dicentric chromosome. If this dicentric were excluded from both daughter nuclei, then one daughter nucleus would contain a rod-X-chromosome and the other, a ring. Male tissue arising as a consequence of such an event would thus be expected to be wild type in about 50% of cases, and to occur frequently as a twin mosaic area consisting in part of male tissue showing the phenotype of the recessives and in part wild-type male tissue. Such twin mosaic areas would be clearly expressed in the abdomens. Only a very small percentage of flies show elimination of the rod-X-chromosome, and twin male-type mosaic areas were not observed. In addition, the first division is characterized by the phenomenon of gonomeric grouping, thus making unlikely synapsis between the two X-chromosomes.

Two-strand double crossing over would not lead to bridge formation, but would give mosaic areas of female tissue. However, whenever the yellow-singed tissue extended into areas in which male and female expression could be differentiated, the tissues marked with the recessive factors were almost always clearly male in nature. In addition, many of the mosaic areas might have been expected to show either the yellow or singed phenotype. Except for single bristle spots such mosaics occur only very rarely. It may therefore be concluded that the great majority of gynandromorphs do not occur as a result of somatic crossing-over.

Various workers have suggested possible explanations of the instability of ring chromosomes in both plants and *D. melanogaster*. These include (1) lagging,[1, 2] (2) lagging as a result of deficiency in the X-chromosome of *D. melanogaster*,[3] (3) sister-strand crossing over[7] and (4) twists in the plane of reproduction.[4, 5] The latter two processes would lead to elimination following the formation of dicentric or interlocked rings and the consequent bridges.

Cytological observations have indicated bridge formation in somatic divisions in flies carrying the ring chromosome.[4] Simple lagging does not lead to bridge formation, and if these cytological observations were to apply to the cleavage stages the first two hypotheses might be ruled out. The increase, following aging, in elimination of the $X^{c2}$-chromosome during the first cleavage stages would offer decisive evidence between (3) and (4) under a specific condition: If it could be shown that the chromosomes of the sperm are split before entry into the egg, then the maternal effect could not be due to an influence on the plane of division. Thus hypothesis (4) could be ruled out. If the chromosomes of the sperm can be shown not to be split, then (3) cannot be distinguished from (4) by this method because the maternal effect could presumably modify either process. The question of the subdivision of chromosomes in the sperm has been discussed by Muller[8] and Auerbach.[9]

The results reported in this paper and discussed above do not include information on spots of one or two bristles which can be found in large numbers on examination of abdomen mounts under 100× magnification. These small spots are quite different in percentages of phenotypic types from the mosaic areas of the gynandromorphs. The small spots are at present being studied in comparison with those produced by typical somatic crossing-over, and the observations will be reported in detail elsewhere.

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[2] Morgan, L. V., "Composites of *Drosophila melanogaster*," *Pub. Carnegie Inst. Wash.* No. 399, 223–296 (1929).

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[4] Braver, G., and Blount, J. L., "Somatic Eliminations of Ring Chromosomes in *Drosophila melanogaster*," *Rec. Gen. Soc. Am.*, **18**, 78 (1949).

[5] Griffen, A. B., and Lindsley, D. L., "The Production of Gynandromorphs through the Use of Unstable Ring Chromosomes in *Drosophila melanogaster*," *Anat. Rec.*, **96**, 59–60 (1946).

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[7] McClintock, Barbara, "The Production of Homozygous Deficient Tissues with Mutant Characteristics by Means of the Aberrant Mitotic Behavior of Ring-Shape Chromosomes," *Ibid.*, **23**, 315–376 (1938).

[8] Muller, H. J., "An Analysis of the Process of Structural Change in Chromosomes of Drosophila," *J. Genetics*, **40**, 1–66 (1940).

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## *THE VIRULENCE OF BIOCHEMICAL MUTANTS OF KLEBSIELLA PNEUMONIAE**

BY E. D. GARBER, ADELINE J. HACKETT AND R. FRANKLIN

NAVAL BIOLOGICAL LABORATORY AND U. S. NAVAL MEDICAL RESEARCH UNIT NO. 1, NAVAL SUPPLY CENTER, OAKLAND, CALIFORNIA

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Virulence may be defined as an expression of a host-parasite relationship.[1] This definition is essentially ecological. The result of an invasion of the host by a parasite depends upon the efficiency of the host's defensive mechanisms relative to the ability of the parasite to proliferate and establish itself. If the parasite does not obtain needed nutrilites from the host, it cannot proliferate. Bacon, Burrows and Yates [2–4] have reported that the decreased virulence of purine-requiring mutants of *Salmonella typhosa* inoculated into the peritoneal cavity of the mouse was due to the absence of purine in the cavity. This observation suggested a procedure for "assaying" specific areas of the host for the presence of amino acids, pyrimidines and purines by means of biochemical mutants of a pathogen. The results of titrations of the virulence of biochemical mutants of *Klebsiella pneumoniae* indicate that such a procedure may be feasible.

*Materials and Methods.*—Mutants of *K. pneumoniae* (strain Kp, Type B) were obtained by means of ultra-violet irradiation followed by penicillin treatment.[5, 6] The M-9 medium[7] was used whenever a synthetic medium was required; Noble agar (Difco) was added to a concentration of 1.5% to prepare solid media. Nutritional requirements were determined auxanographically.

Strain Kp is virulent for the Namru strain mice[8] when inoculated either

intraperitoneally (LD-50 = *ca.* 20 cells) or intranasally (LD-50 = *ca.* $8 \times 10^3$ cells). The Namru strain of mice was used throughout. The virulence of the biochemical mutants was determined by inoculating mice weighing approximately 20 g. intraperitoneally with 0.2 ml. or intranasally with 0.05 ml. of a saline suspension of thoroughly washed cells. Aliquots

TABLE 1

RELATION BETWEEN VIRULENCE AND NUTRITIONAL REQUIREMENTS OF BIOCHEMICAL MUTANTS OF *Klebsiella pneumoniae* INOCULATED INTO MICE EITHER INTRAPERITONEALLY OR INTRANASALLY

| | | ROUTE OF INOCULATION | | | |
|---|---|---|---|---|---|
| | | INTRAPERITONEAL | | INTRANASAL | |
| MUTANT | REQUIREMENT | DOSAGE | DEAD/TOTAL | DOSAGE | DEAD/TOTAL |
| Wild | None | $3 \times 10^2$ | 2/5 | $7 \times 10^3$ | 0/5 |
| | | $3 \times 10^3$ | 5/5 | $7 \times 10^4$ | 3/5 |
| | | $1 \times 10^4$ | 9/10 | $3 \times 10^5$ | 9/10 |
| | | $1 \times 10^5$ | 10/10 | $3 \times 10^6$ | 10/10 |
| 34 | Threonine | $1 \times 10^4$ | 10/10 | $3 \times 10^5$ | 10/10 |
| | | $1 \times 10^5$ | 10/10 | $3 \times 10^6$ | 10/10 |
| 2 | Tyrosine | $1 \times 10^4$ | 9/10 | $3 \times 10^5$ | 8/10 |
| | | $1 \times 10^5$ | 10/10 | $3 \times 10^6$ | 10/10 |
| 40 | Methionine | $1 \times 10^4$ | 9/10 | $3 \times 10^5$ | 10/10 |
| | | $1 \times 10^5$ | 10/10 | $3 \times 10^6$ | 10/10 |
| 27 | Leucine | $1 \times 10^4$ | 9/10 | $3 \times 10^5$ | 8/10 |
| | | $1 \times 10^5$ | 10/10 | $3 \times 10^6$ | 10/10 |
| 17 | Histidine | $1 \times 10^4$ | 10/10 | $3 \times 10^5$ | 10/10 |
| | | $1 \times 10^5$ | 10/10 | $3 \times 10^6$ | 10/10 |
| 22 | Uracil | $3 \times 10^2$ | 1/5 | $7 \times 10^3$ | 1/5 |
| | | $3 \times 10^3$ | 4/5 | $7 \times 10^4$ | 2/5 |
| 2711 | Guanine | $3 \times 10^2$ | 0/10 | $8 \times 10^3$ | 0/10 |
| | | $3 \times 10^3$ | 0/10 | $8 \times 10^4$ | 0/10 |
| | | $3 \times 10^4$ | 0/10 | $8 \times 10^5$ | 0/10 |
| 58 | Adenine | $3 \times 10^2$ | 0/5 | $7 \times 10^3$ | 0/5 |
| | | $3 \times 10^3$ | 0/5 | $7 \times 10^4$ | 0/5 |
| | | $1 \times 10^4$ | 0/10 | $3 \times 10^5$ | 0/10 |
| | | $1 \times 10^5$ | 0/10 | $3 \times 10^6$ | 0/10 |
| 58-01 | None | $1 \times 10^2$ | 8/10 | $3 \times 10^3$ | 4/10 |
| | | $1 \times 10^3$ | 10/10 | $3 \times 10^4$ | 7/10 |
| | | $1 \times 10^4$ | 9/10 | $3 \times 10^5$ | 9/10 |
| | | $1 \times 10^5$ | 10/10 | $3 \times 10^6$ | 10/10 |
| 58-02 | None | $1 \times 10^2$ | 2/10 | $3 \times 10^3$ | 1/10 |
| | | $1 \times 10^3$ | 3/10 | $3 \times 10^4$ | 0/10 |
| | | $1 \times 10^4$ | 6/10 | $3 \times 10^5$ | 9/10 |
| | | $1 \times 10^5$ | 9/10 | $3 \times 10^6$ | 9/10 |

of a single bacterial suspension were used for intraperitoneal and intranasal inoculations; viable cell counts were made by plating suitable dilutions of the suspension on nutrient agar.

Cultures obtained from the peritoneal fluid of animals succumbing after an intraperitoneal inoculation and from lung tissue of animals succumbing

after an intranasal inoculation were tested to determine whether the specific requirement was still present. In all tests, only thoroughly washed cells were used. Animal experiments were terminated 10 days after inoculation, since no deaths occurred after this period.

*Results.*—Mice were inoculated with the following biochemical mutants: threonine-less, tyrosine-less, methionine-less, leucine-less, and histidine-less (amino-acids), uracil-less (pyrimidine), guanine-less, adenine-less and "complex" purine-less (purines). The parental strain (Kp) was inoculated as a control. The results summarized in table 1 indicate that the amino-acid-requiring mutants and the pyrimidine-requiring mutant were as virulent as the parental strain when inoculated either intraperitoneally or intranasally. In all cases, cultures recovered from the mice were mutants indicating that reversion to wild type had not occurred in the host. The purine requiring mutants, however, were avirulent when inoculated by either route.

TABLE 2

THE EFFECT OF INTRAPERITONEAL INOCULATIONS OF THE "COMPLEX" PURINE-LESS MUTANT, 27139, OF *Klebsiella pneumoniae* AND EITHER HYPOXANTHINE OR A PURINE POOL*

| EXPERIMENT | TREATMENT | TOTAL DOSAGE | DEAD/TOTAL |
|---|---|---|---|
| I | 27139 | $1.3 \times 10^5$ | 0/10 |
| | Hypoxanthine | 50 mg. | 0/10 |
| | 27139 plus hypoxanthine | $1.3 \times 10^5$ 50 mg. | 3/10 |
| II | 27139 | $8 \times 10^6$ | 0/10 |
| | Purine pool | 24 mg. | 0/10 |
| | 27139 plus purine pool | $8 \times 10^6$ 24 mg. | 10/10 |

* 15 mg. hypoxanthine, 3 mg. xanthine, 3 mg. adenine, 3 mg. guanine.

Small pads of filter paper containing peritoneal fluid from normal mice were added to the surface of pour plates of the biochemical mutants in minimal agar. Peritoneal fluid supplied the requirements for the amino-acid and pyrimidine dependent mutants but not for the purine dependent mutants. The avirulence of purine-requiring mutants of *K. pneumoniae* inoculated intraperitoneally into the mouse parallels the observations of Bacon, *et al.*,[3, 4] with *S. typhosa*.

If the avirulence of the adenine-less mutant was due to its failure to proliferate in an adenine deficient site, reversion to adenine independence would be expected to lead to virulence. Two reversions were obtained in a population of *ca.* $10^{11}$ viable cells plated in minimal medium. Both reversions, 58-01 and 58-02, were virulent when inoculated into mice either intraperitoneally or intranasally (see table 1).

The "complex" purine-less mutant, 27139, requires either guanine, ade-

nine, xanthine or hypoxanthine for growth. Since the response of the mutant in terms of growth *in vitro* was greater for a pool of these 4 purines compared with the response for any one of the purines, the effect of inoculating graded dosages of hypoxanthine or a pool of the 4 purines into the peritoneal cavity of mice previously inoculated by the same route with mutant 27139 was determined. Suitable controls were used. The results summarized in table 2 indicate that the response to hypoxanthine was less severe than the response to the purine pool. Cultures recovered from the peritoneal cavity and lungs of the dead mice were mutant indicating that reversions to the wild type were not responsible for the response. Moribund mice were sacrificed and only mutant cultures were recovered. No deaths were observed in the control groups.

*Discussion.*—The loss of virulence of purine-requiring mutants of *S. typhosa* and *K. pneumoniae* reflects the inability of these mutants to grow in the peritoneal cavity of the mouse. Bacon, *et al.*[3, 4] demonstrated in the case of *S. typhosa* that the decreased virulence of purine-requiring mutants was due to the absence (or perhaps unavailability) of purines in the peritoneal cavity of the mouse. These observations suggest that purine-requiring mutants of any pathogen may display decreased virulence when inoculated intraperitoneally into the mouse. That this situation may be extended to the mouse lung is indicated by the results obtained with the purine-requiring mutants of *K. pneumoniae*.

Since the addition of the specific requirement, purine, permitted the "complex" purine-less mutant, 27139, to proliferate in the host, resulting in the death of the host, it is reasonable to assume that the avirulence of the mutant had been due to the absence or unavailability of purine in the peritoneal cavity of the mouse.

Biochemical mutants of pathogens may be used to explore the biochemistry of sites of inoculation or localization in susceptible hosts. In its most simple form, a pathogen causing death rather than infection would be desirable. Death following inoculation of a mutant with a specific requirement would indicate the presence of the specific requirement. Decrease or loss of virulence, however, need not indicate absence of the requirement. The requirement may not be satisfied due to its extremely low concentration or the presence of antagonists or antimetabolites, for example.

*Summary.*—Five amino-acid and one pyrimidine-requiring mutants of *Klebsiella pneumoniae* were as virulent as the parental strain when inoculated into mice either intraperitoneally or intranasally. Purine-requiring mutants were avirulent when inoculated by either route. Virulence was restored with reversion to purine independence. The addition of purine to the peritoneal cavity of mice inoculated with a purine-requiring mutant inoculated by the same route resulted in death. The use of bio-

chemical mutants of a pathogen to explore the biochemistry of sites of inoculation or localization in susceptible hosts is suggested.

* This work is supported in part by a contract between the University of California, Department of Bacteriology, and the Office of Naval Research. The opinions contained in this report are not to be construed as reflecting the views of the Navy Department or the Naval service at large.

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## *THE ROLE OF OXYGEN CONCENTRATION IN DETERMINING THE EFFECTIVENESS OF X-RAYS ON THE ACTION OF A SPECIFIC GENE IN DROSPHILA MELANOGASTER*

BY BENTLEY GLASS AND HENRY L. PLAINE

DEPARTMENT OF BIOLOGY, THE JOHNS HOPKINS UNIVERSITY

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The discovery by Thoday and Read[1] that the oxygen concentration at the time of treatment with x-rays profoundly affects the production of chromosome breaks in *Vicia faba* has resulted in a great deal of work on this and related phenomena. Geneticists have been particularly interested in the effect of anoxia on gene and chromosome mutation and the light which is thereby thrown on the nature of the mutation process.[2–12] Other workers have been equally interested in the bearing of the finding on the protection of animals from irradiation damage and in the mechanism of the protective action exerted by antioxidants.[13–15] Since it had been found by the authors that x-ray treatments of embryos or early larval stages of *Drosophila melanogaster* will block the action of a specific suppressor gene and allow the normally present but suppressed mutant gene "erupt" to manifest itself in the adult stage,[16–18] it seemed of obvious interest to test the effect of oxygen concentration upon the action of the x-rays on this developmental process.

Two genetic stocks of *D. melanogaster* were used for comparison in the study: (1) suppressor-erupt stock, *Su-er bw; st er*, which had been made isogenic and had been maintained thereafter by close inbreeding; and (2)

a wild-type stock, Oregon-R. The latter had previously been found (unpublished results), after x-ray treatment of the embryos with 1000 r units, to yield 3.4 per cent of adults with the extreme erupt manifestations, and 38.1 per cent of adults with facet disarrangements of the eyes, which may or may not have been a weaker manifestation of the erupt phenotype. This low frequency of erupt contrasts strikingly with the previously found frequency of erupt in adults developing from x-rayed embryos of the suppressor-erupt stock, namely, 65.9 per cent of extreme erupt, 95.7 per cent of all grades combined.[18] It was supposed that the effect of the oxygen concentration could best be demonstrated by using two stocks of such contrasting character in respect to erupt.

Eggs were collected on banana-cornmeal agar over a 2-hr. period and were x-rayed at 10 ± 1 hr. of embryonic development. All series received

TABLE 1

THE EFFECT OF OXYGEN CONCENTRATION ON THE EFFECTIVENESS OF X-RAYS IN BLOCKING THE SUPPRESSOR OF ERUPT IN THE *Su-er bw; st er* STOCK

| | | TOTAL CLASSIFIED | PHENOTYPE OF EYES: NORMAL | WEAK ERUPT | EXTREME ERUPT | % NORMAL | % EXTREME ERUPT |
|---|---|---|---|---|---|---|---|
| | | | A. Not X-rayed | | | | |
| $N_2$ | 1 series[a] | 216 | 179 | 37 | 0 | 82.9 ± 2.6 | 0.0 |
| Air | 1 series | 500 | 454 | 46 | 0 | 91.8 ± 1.2 | 0.0 |
| $O_2$ | 1 series | 1364 | 1291 | 72 | 1 | 94.6 ± 0.6 | 0.1 |
| | | | B. X-rayed 1000 R | | | | |
| $N_2$ | 1 series[a] | 233 | 142 | 71 | 20 | 60.9 ± 3.2 | 8.6 ± 1.8 |
| | 3 series | 918 | 739 | 136 | 43 | 80.5 ± 1.3 | 4.7 ± 0.7 |
| 10% $O_2$ / 90% $N_2$ | 2 series | 476 | 210 | 48 | 218 | 44.0 ± 2.3 | 45.8 ± 2.3 |
| Air | 1 series[a] | 41 | 3 | 7 | 31 | 7.3 ± 4.1 | 75.6 ± 6.7 |
| | 3 series | 435 | 37 | 34 | 364 | 8.5 ± 1.3 | 83.7 ± 1.8 |
| $O_2$ | 2 series | 255 | 5 | 31 | 219 | 2.0 ± 0.9 | 85.9 ± 2.2 |

[a] At embryonic age 5 ± 1 hr. instead of 10 ± 1 hr.

1000 r units (60 kv., 15 ma., distance 12 in., 80–100 r per minute). Before and after the x-ray treatments all were kept at 25 ± ½°C.; during the treatments the room temperatures were between 24° and 26.5°C. The eggs to be treated were irradiated within a specially made lucite chamber with an opening so placed that dosage readings could be made with an ionization chamber at precisely the point where the eggs were subsequently placed. At the time of treatment in a gas of particular composition, this opening and the side through which the eggs are introduced were both sealed. The chamber possesses inlet and outlet tubes for the flow of gas, which was introduced under moderate pressure. In the first tests, both inlet and outlet tubes were then shut off with screw clamps. After an immediate test to exclude leakage, the eggs were x-rayed. Subsequently,

when it was found that exposure to the various gases used had in itself no effect on the frequency or manifestation of erupt (see tables 1, 2), and that the gas needed to be present only at the time of x-ray irradiation, a simple flow method was substituted. The gas was allowed to flow through the chamber during treatment, just enough gas being passed through before commencement of the treatment to ensure that all air in the chamber had been replaced. The results obtained by either method were identical. The eggs were kept after treatment in petri dishes for 24 hr., until a count of the percentage of eggs hatching had been completed. They were then transferred to regular Drosophila culture bottles, and were kept at 25°C. until the adults eclosed.

The gases used were as follows: pure nitrogen; 10 per cent $O_2$/90 per cent $N_2$; air (approximately 20% $O_2$); pure $O_2$. The pure oxygen and nitrogen and the 10% $O_2$ mixture were from commercially supplied cylinders with low percentages of impurities.

TABLE 2

THE EFFECT OF OXYGEN CONCENTRATION ON THE EFFECTIVENESS OF X-RAYS IN REVEALING ERUPT IN THE OREGON-R STOCK

| | | TOTAL CLASSFIED | PHENOTYPE OF EYES: NORMAL | WEAK ERUPT | EXTREME ERUPT | % NORMAL | % EXTREME ERUPT |
|---|---|---|---|---|---|---|---|
| A. Not X-rayed | | | | | | | |
| $N_2$ | 1 series | 500 | 500 | 0 | 0 | 100.0 | 0.0 |
| Air | 1 series | 500 | 498 | 2 | 0 | 99.6 | 0.0 |
| $O_2$ | 1 series | 500 | 499 | 1 | 0 | 99.8 | 0.0 |
| B. X-rayed 1000 R | | | | | | | |
| $N_2$ | 2 series | 698 | 684 | 10 | 4 | 98.0 ± 0.5 | 0.57 ± 0.3 |
| 10% $O_2$ / 90% $N_2$ | 2 series | 536 | 504 | 23 | 9 | 94.0 ± 1.0 | 1.68 ± 0.6 |
| Air | 3 series | 431 | 303 | 113 | 15 | 70.3 ± 2.2 | 3.48 ± 0.9 |
| $O_2$ | 2 series | 246 | 162 | 64 | 20 | 65.9 ± 3.0 | 8.14 ± 1.7 |

Table 1 summarizes the results of the effect of oxygen concentration on the action of x-rays upon the suppressor-erupt stock, and table 2 presents the results for the Oregon-R wild-type stock. In figure 1 a graphic comparison of the two sets of results is given. It will be seen that the effect of oxygen concentration is apparent in both stocks, although at very different levels of incidence of erupt. The most striking difference occurs in the interval between 20 per cent $O_2$ (air) and 0 per cent $O_2$ (100% $N_2$). In the Oregon-R stock the percentage of extreme erupt is reduced from 3.48 to 0.57 per cent in the absence of oxygen, the difference being statistically highly significant ($\chi^2 = 13.66$; d. f. = 1; $P < 0.001$). In the suppressor-erupt stock the difference is much more striking, since the frequency of extreme erupt drops from 82.98 per cent in air to 5.47 per cent in the absence of oxygen. At 10 per cent $O_2$, both stocks showed frequencies of extreme

erupt almost exactly halfway between the respective frequencies found in air and in the absence of oxygen. It appears that the effect of x-rays in pure $O_2$ is not much greater than in air, although there is some increase. Whether the curve between 20 and 100 per cent $O_2$ is actually nearly flat cannot be stated, however, until intermediate concentrations have been tested.

General viability is also strongly affected by the presence or absence of $O_2$ at the time of x-ray treatment (tables 3, 4). When the eggs are x-rayed

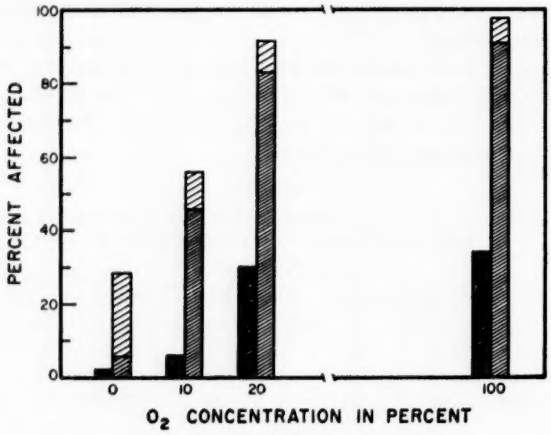


FIGURE 1

The percentage of flies manifesting erupt after being x-rayed as embryos in the indicated concentrations of oxygen. The lower, darker portion of each column represents the frequency of individuals classified as extreme erupt; the upper part of each column represents the frequency of individuals with a weak manifestation of erupt. At each concentration, the column to the left represents the data for the Oregon-R wild-type stock, the column to the right the data for the suppressor-erupt stock (*Su-er bw; st er*).

at 10 ± 1 hr. after laying, there is no significantly increased mortality during the embryonic period. The percentage of eggs hatching is variable, ranging from 78.3 to 96.6 per cent in different series, but it is doubtful whether this is attributable to the effect of the x-ray treatment in a particular $O_2$ concentration, rather than to extrinsic factors. On the other hand, the mortality of the larval and pupal stages shows a steady increase (i.e., a decrease of adults eclosing) as the oxygen concentration at the time of x-ray treatment is increased. This is to be seen in the columns of tables 3 and 4 headed "Adults Eclosing as Percentage of Eggs Hatched"; that is,

the conclusion applies to both the stocks used. Only the frequency of hatching in the three series of the suppressor-erupt stock treated in pure $N_2$ is out of line, creating a discrepancy which further work must explain. Apart from this discrepancy, the data clearly show that for both stocks x-rays have an increasingly fatal effect as the oxygen concentration increases. In pure oxygen an x-ray treatment of 1000 r units kills approximately 95.0 per cent of the suppressor-erupt individuals and 70 per cent of the Oregon-R wild-type individuals which have been treated at the age of 10 ± 1 hr.

On the other hand, treatment with x-rays at the same age and with the same dose, but in pure nitrogen, appears to kill relatively few, if any, individuals during the larval-pupal period. Not only is this true, but also

TABLE 3

EFFECTS UPON DEVELOPMENT IN THE SUPPRESSOR-ERUPT STOCK, OF X-RAY TREATMENT IN VARIOUS CONCENTRATIONS OF OXYGEN

| | | TOTAL EGGS LAID | EGGS HATCHED, % | ADULTS ECLOSING AS PERCENTAGE OF EGGS HATCHED | ADULTS ECLOSING THROUGH 9TH DAY AS PERCENTAGE OF ADULTS | DAY OF EARLIEST ECLOSION |
|---|---|---|---|---|---|---|
| | | A. Not X-rayed | | | | |
| $N_2$ | 1 series[a] | 274± | 95.0 | 87.5 | 56.0 | 9th |
| Air | 1 series | 540 | 97.2 | 95.2 | 40.0 | 9th |
| $O_2$ | 1 series | 1400± | 98.3 | 99.0 | 0.0 | 10th |
| | | B. X-rayed 1000 R | | | | |
| $N_2$ | 1 series[a] | 291 | 91.0 | 89.0 | ... | ... |
| | 3 series | 1905 | 82.1 | 58.5 | 17.4 | 9th |
| 10% $O_2$ / 90% $N_2$ | 2 series | 750 | 78.5 | 80.8 | 0.0 | 11th |
| Air | 1 series[a] | 410 | 46.0 | 21.7 | ... | ... |
| | 3 series | 2477 | 87.5 | 20.1 | 0.0 | 10th |
| $O_2$ | 2 series | 2900± | 88.0 | 6.3 | 0.0 | 10th–11th |

[a] At embryonic age 5 ± 1 hr. instead of 10 ± 1 hr.

the development, which is greatly extended in duration following treatment with x-rays in oxygen, becomes of normal duration after x-ray treatment in nitrogen, or may even be faster than normal in some individuals (see table 4, column "Day of Earliest Eclosion").

In the third larval instar of individuals which have been treated at 10 ± 1 hr. (embryonic age) with 1000 r units of x-rays in air, a high incidence of melanotic tumors is to be observed. These tumors are free-floating in the body fluids and resemble in structure those studied by Russell,[19] and which she found to be transplantable and benign. In the Oregon-R stock, in which untreated controls rarely if ever develop a melanotic tumor, we have observed an incidence in x-rayed individuals of 10.0 per cent. Hartung[20] found an incidence of somewhat less than 8.0 per cent of individuals with

tumors in his $T_2$ stock at an x-ray dose of 1000 r units; but as high as 32.9 per cent in his $T_3$ strain. In our suppressor-erupt stock we find an incidence of 82.0 per cent (N = 1400) in x-rayed individuals, and many of the individuals have multiple tumors, as many as 6 or 8 per individual in some cases. The frequency of individuals with tumors, like the frequency of manifestation of erupt, varies in both stocks with the concentration of oxygen present at the time of treatment. Thus, for example, x-raying the Oregon-R individuals in pure nitrogen leads to no production of tumors at all. Our complete data on the dependence of tumor induction by x-rays upon the concentration of oxygen at the time of irradiation will be reported fully in another paper.

There is no clear indication that the high mortality in the stocks irradi-

TABLE 4

EFFECTS UPON DEVELOPMENT IN THE OREGON-R STOCK, OF X-RAY TREATMENT IN VARIOUS CONCENTRATIONS OF OXYGEN

| | | TOTAL EGGS LAID | EGGS HATCHED, % | ADULTS ECLOSING AS PERCENTAGE OF EGGS HATCHED | ADULTS ECLOSING THROUGH 9TH DAY AS PERCENTAGE OF ADULTS | DAY OF EARLIEST ECLOSION |
|---|---|---|---|---|---|---|
| | | A. Not X-rayed | | | | |
| $N_2$ | 1 series | 500 | 94.0 | 92.1 | 39.3 | 9th |
| Air | 1 series | 500 | 96.6 | 93.4 | 21.3 | 9th |
| $O_2$ | 1 series | 1500± | 97.4 | 99.5 | 0.0 | 10th |
| | | B. X-rayed 1000 R | | | | |
| $N_2$ | 2 series | 1000 | 92.8 | 75.3 | 10.4 | 8th |
| 10% $O_2$ / 90% $N_2$ | 2 series | 1000 | 96.6 | 55.5 | 0.0 | 11th |
| Air | 2 series | 800 | 92.5 | 41.9 | 0.0 | 12th |
| $O_2$ | 2 series | 1000 | 78.3 | 31.4 | 0.0 | (*a*) 10th<br>(*b*) 13th |

ated with x-rays in air or pure oxygen is caused by the development of the melanotic tumors. The data merely indicate that both mortality and incidence of tumors increase in a parallel manner as the oxygen concentration at the time of irradiation increases. Individual larvae with multiple tumors may pupate normally and emerge as viable adults with melanomas clearly visible within the abdominal cavity.

The effect upon mortality is in other respects like that reported by Limperos[14] in rats and mice irradiated in atmospheres from almost pure oxygen down to 5 per cent oxygen. The mortality was found to be much higher with the higher oxygen concentrations. This was also found by Hollaender, Stapleton and Martin[21] to hold true of *Escherichia coli* treated with x-rays in various tensions of oxygen.

On the other hand, Baker and Sgourakis,[6] who tested the effect of x-rays

in various concentrations of oxygen on the induction of sex-linked lethals in Drosophila, found that the viability of adult males which had been treated with 5000 r units was lower after treatment in $N_2$ than after treatment in $O_2$. Hence the embryo stage of Drosophila, according to our experiments, differs in this respect from the adult males. But Baker and Sgourakis also found that, conversely to the effect on the viability of the treated adult males, the number of offspring produced was reduced far more when the males had been x-rayed in $O_2$ than when they were x-rayed in $N_2$. This decrease in fecundity they attributed to the induction of dominant mutations and chromosome aberrations in the treated spermatozoa, since they found no evidence of any failure of the treated males to mate with females or any loss of motility on the part of the treated spermatozoa. In our experiments, however, the mortality of the individuals treated while embryos appears to be more likely due to some cytoplasmic effect, (1) inasmuch as the mortality parallels the effect on the action of the suppressor-erupt gene, which has been shown not to involve mutation;[16, 18] and (2) inasmuch as actual death occurs mostly in the late third instar larvae or in the pupae, whereas dominant lethal mutations and inviable chromosome aberrations are known to kill mostly in the egg stage.[22–24] The same arguments apply to the induction of the melanotic tumors. The action of the x-rays in $O_2$ elicits certain specific responses which vary in degree according to the genetic constitution of the strain treated. There is no reason to suppose the genes themselves to be affected; their action is simply modified, either by destruction of substrate or gene product. The action, as in the production of phenocopies when developing individuals are treated with a particular agent at a particular stage of development, is highly specific. In the present case, there is as yet no evidence that more than a single gene's action, namely, that of the suppressor-erupt gene, is affected by the irradiation with x-rays in various oxygen tensions. It will, of course, be necessary to conduct more refined genetic experiments, combined with the x-ray treatments in $O_2$, to prove conclusively that the effect upon mortality and the induction of the melanotic tumors are invariably associated with the presence of the erupt mutant and its x-ray-sensitive suppressor gene.

At the present time, there is much discussion of the nature of radiation injury to animals and the protective action of various chemical substances against radiation injury. In brief, the prevailing theory is that ionizing radiation produces highly oxidizing free radicals in water, and that the concentration of these is vastly increased when $O_2$ is present in solution.[25–27] Hydrogen peroxide, and perhaps organic peroxides, result. These have been shown to act as mutagens for conidial spores of Neurospora[28] and for cells of *E. coli* and other bacteria.[29] But such agents would be expected to have a direct action on any reduced substances in the cytoplasm, in addition to any action they might have upon the chromosomes

or genes. Hence substances that act as antioxidants should protect against radiation effects, as indeed in many cases they do. Barron[30] has reported extensive investigations of the protective action of sulfhydryl compounds upon irradiated enzymes. Cysteine and glutathione,[31] thiourea,[32] and even cyanide[27] protect animals (rats or mice) against ionizing radiations. Tumor cells in the animal or in tissue culture may also be protected in the same way.[33, 27] Bacteria are protected against the lethal action of x-rays by sodium formate, ethanol and various glycols[21]; and mice by para-aminopropiophenone and by sodium nitrite.[34] Any of these protective substances must be present at the time of irradiation to be effective. On the other hand, the almost universal presence in tissues of catalase or other peroxide-destroying enzymes makes one wonder how peroxides could persist long enough, when generated in protoplasm, to reach vital targets and produce such effects.

The great promise of the present system is that it provides, on account of the great sensitivity of the action of the suppressor-erupt gene to x-rays in $O_2$, a most convenient and suitable system for testing such theories, for assaying the effects of radiation and chemical agents and of protective substances, and for relating gene action to the genesis of tumors.

*Summary.*—X-ray treatments of embryos of the suppressor-erupt stock block the action of a specific suppressor gene (*Su-er*) and allow the suppressed mutant gene erupt (*er*) to manifest itself in the adult stage. This stock also shows a high incidence of melanotic tumors (81.7 per cent) induced by x-rays. When embryos were irradiated with 1000 r units at 10 ± 1 hr. in concentrations of 0 per cent $O_2$, 10 per cent $O_2$, 20 per cent $O_2$, and pure oxygen, there was, with increasing oxygen concentration, (1) increased inhibition of the action of the suppressor gene, with corresponding increase in the expression of erupt; (2) increased mortality in the larval and pupal stages; (3) increased duration of development, with delayed pupation; and (4) increased incidence of tumors as well as increased number of tumors per affected larva. There is no observable effect when embryos are exposed to various $O_2$ concentrations without being x-rayed. The incidence of erupt and the incidence of tumors both increase significantly and linearly from 0 to 20 per cent $O_2$, above which concentration there is only a small, though significant, further increase. The same treatment applied to an Oregon-R inbred strain gives similar results but at a much lower level. Mortality, extended and delayed development, and induction of melanomas all parallel the effects of x-rays on the suppression of erupt at given oxygen tensions; but further work will be required to make clear the nature of the relation between the suppressor of erupt and the parallel effects.

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# A GENETIC ANALYSIS OF THE ADAPTIVE VALUES OF POPULATIONS*

By Bruce Wallace and J. C. King

The Biological Laboratory, Cold Spring Harbor, N. Y

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Every species of organisms exists because it perpetuates itself in a given ecological niche more effectively than do its competitors. It has been pointed out by Gause[1] that no two species can permanently occupy the same niche because one of them will always prove to be more efficient than the other and the less efficient one will eventually be crowded out. Hence, where two closely related species co-exist, each specializes in a slightly different habitat.

Within the same species, wherever individuals from populations of different origin come together, the problem of competition is complicated by interbreeding and the mingling of genetic material. But when interbreeding is prevented by experimental control, it can be generally shown that two populations need not be exactly equal in the efficiency with which they perpetuate themselves. Such efficiency of perpetuation may be termed adaptive value and its determination is of primary importance in the study of populations. Numerous methods, usually measures of selected physiological traits obviously relevant to the maintenance of the population, have been used for estimating adaptive values. Some estimates for five experimental populations of *Drosophila melanogaster* have previously been reported by Wallace and King,[2] and Wallace.[3] The present paper deals with some attempts at identifying the determinants of these adaptive values.

The technique used for obtaining estimates of the adaptive values for these more detailed analyses was the genetic test described in the above references. Through the use of a stock of flies carrying a genetically marked second chromosome (*Cy L*) samples of second chromosomes were isolated from the populations. The effect of these sampled chromosomes on the viability of individuals carrying them in the homozygous condition or in heterozygous combinations with other chromosomes from the same population was determined. These viabilities were determined in relation to the *Cy L*/ + flies hatching in the same cultures and in both cases ranged from complete lethal (0% wild type) to "normal" (approximately the 33.3% wild flies of the theoretically expected 2*Cy L*/+:1 +/+ ratio). This technique then, resulted in the assignment of characteristic viabilities to certain chromosomes on the basis of the homozygous tests and the determination of viabilities of combinations of these chromosomes. In order to get a comprehensive cross-section of the populations, limited data on a great many combinations were collected rather than enormous data on a

few; these studies include over 6000 heterozygous combinations, and for over 2700 of these the corresponding homozygous tests are available.

The adaptive values of the populations were estimated from the average frequencies of wild flies in all heterozygous tests for each population. These average frequencies proved to be characteristic for each population (table 1) and from them the adaptive values were calculated. These adaptive values remained constant over a long period of time in spite of profound genetic changes revealed by the homozygous tests.

In estimating the adaptive values by this technique all the tests were included in the calculations. Each test culture gave an estimate of the viability of individuals possessing a given genotype (second chromosome only) and the use of all tests guaranteed the inclusion of the widest possible array of genotypes to be found within the populations. Certain of these tests resulted in lethal or semilethal combinations; either no wild flies or less than half of those expected appeared. These latter tests indicated that occasionally certain genotypes were missing from the populations because of their lethal nature.

TABLE 1

THE AVERAGE FREQUENCY OF WILD FLIES IN *All* AND IN *Normal* HETEROZYGOUS TESTS OF THE EXPERIMENTAL POPULATIONS. $s-a$ AND $s-n$ EQUAL THE DIFFERENCES FROM THE VIABILITY OF NORMAL HETEROZYGOTES OF POPULATION 1 TO ALL AND NORMAL HETEROZYGOTES, RESPECTIVELY, OF THE OTHER POPULATIONS. $\% = (s-n)/(s-a)$

| POP. | ALL | NO. | NORMAL | NO. | $s-a$ | $s-n$ | % |
|---|---|---|---|---|---|---|---|
| 1 | 35.06 | 1454 | 35.16 | 1450 | .. | .. | .. |
| 3 | 34.14 | 1181 | 34.19 | 1179 | 1.02 | 0.97 | 95 |
| 5 | 31.85 | 1007 | 32.23 | 994 | 3.31 | 2.93 | 89 |
| 6 | 32.48 | 1326 | 32.66 | 1318 | 2.68 | 2.50 | 93 |
| 7 | 33.40 | 1350 | 33.44 | 1348 | 1.76 | 1.72 | 98 |

It is widely—if often tacitly—assumed that a population consists of a majority of identically "normal" individuals and a varying—from population to population—proportion of "subnormal" individuals whose presence determines the adaptive value of the population. Here we have extensive data on the viabilities of large numbers of different genotypes from several populations. It appears that these data can throw light on the influence of lethal and semilethal genotypes on the adaptive value of the whole population. Table 1 gives the average frequency of wild flies in all and in the "normal" (non-lethal, non-semilethal) heterozygous tests of the five populations. The highest average frequency in the table is that of normal individuals of population 1; this frequency can be regarded, then, as a standard of viability. To determine the role of drastically weakened individuals of population 3, for instance, in determining the adaptive value of that population, one compares the amount by which the average of all cultures of this population falls short of the standard and the amount by which the

average of the normal cultures is deficient. Actually, 95% of the total deficit in the average frequency of all cultures can be ascribed directly to those genotypes with normal viability; the inclusion of the lethals and semilethals adds only another 5% to the deficit already present. Lethals and semilethals have their greatest effect in population 5, a population of 100–1000 adult individuals that received 2000r per generation for some 40 generations. In population 7 those genotypes with very poor viabilities lowered the adaptive value only an additional 2%. In each population the data indicate that the outstanding determinant of the adaptive value of that population is the large bulk of normal individuals; the obviously non-normal ones have a very minor role in establishing the adaptive value.

The properties of a population of interbreeding diploid organisms at any given time can be best estimated by summing the attributes of the indi-

TABLE 2

VIABILITIES OF FLIES HETEROZYGOUS FOR SECOND CHROMOSOMES WITH DRASTIC OR NON-DRASTIC EFFECTS WHEN HOMOZYGOUS AS EXPLAINED IN TEXT. BOTH THE MEAN AND 95% FIDUCIAL LIMITS OF VIABILITIES ARE GIVEN. RANK OF EACH MEAN IS SHOWN. NUMBERS IN PARENTHESES ARE NUMBER OF COMBINATIONS STUDIED

| POP. | 2-DRASTIC | 1-DRASTIC | NO-DRASTIC |
|---|---|---|---|
| 1 | 35.32 (33.74−36.90)<br>15 (38) | 34.74 (34.18−35.30)<br>13 (223) | 34.75 (34.31−35.19)<br>14 (352) |
| 3 | 33.66 (32.44−34.88)<br>8 (28) | 34.43 (33.91−34.95)<br>10 (216) | 34.53 (34.09−34.97)<br>11 (292) |
| 5 | 31.62 (31.08−32.16)<br>1 (239) | 32.38 (31.86−32.90)<br>3 (235) | 32.03 (31.03−33.03)<br>2 (54) |
| 6 | 32.54 (32.00−33.08)<br>4 (231) | 33.15 (32.63−33.67)<br>7 (243) | 34.54 (33.40−35.68)<br>12 (54) |
| 7 | 32.69 (31.11−34.27)<br>5 (29) | 32.98 (32.38−33.58)<br>6 (163) | 33.77 (33.37−34.17)<br>9 (339) |
| Rank total | 33 | 39 | 48 |

viduals alive at that moment but the enduring characteristics of the group from one generation to another are determined by the genetic pool which the individuals represent only as transitory combinations. The pool—except as changed by selection or genetic drift—remains the same; the individuals change. A corresponding situation is found in a bridge game where the deck of cards is constant but the hands change from one deal to the next. We can know the value of a bridge hand from a knowledge of the cards it contains only within limits. Much depends upon the position of other cards in other hands and the skill of the player in bidding and selecting his plays. Can we predict from the behavior of a chromosome in homozygous condition what its contribution will be to the adaptive value of the population where in all likelihood it will be in heterozygous condition? By studying each second chromosome, whose behavior in homozygous condition is

known, in combination with two other randomly chosen second chromosomes, it was possible to get some measure of the contributions made by different types of chromosomes to the adaptive values of the different populations.

For purposes of analysis, the second chromosomes were divided into two classes on the basis of viabilities of individuals carrying them in the homozygous condition: "drastic" chromosomes that permitted fewer than half of the homozygous individuals to survive in the test cultures, and "normal" chromosomes that permitted more than half the expected number to survive. The heterozygous combinations were made before the nature of the chromosomes was known; consequently, the frequency of the various types of combinations approached that found in the populations. For convenience, one can designate the various combinations as "2-drastic," "1-drastic" and "no-drastic" or "normal."

Table 2 gives the average frequencies of wild type flies found in heterozygous test cultures of the three types of combinations. The limits of the 95% confidence interval (± 2 sigmas) and the number of combinations studied in each case are also given. Only the combinations that gave heterozygotes of normal viability are included in this table; lethal and semilethal heterozygous combinations always arose from 2-drastic combinations. From an inspection of the table it is clear that we are operating very close to the limit of statistical resolution; the confidence intervals (even though the standard error in some cases is less than 0.3%) are large relative to the differences found between populations or between different categories within populations. Nevertheless, considerable information can be gleaned from the figures.

The mean viabilities of the three types of combinations are not identical. There is a tendency for no-drastic combinations to yield higher frequencies of wild type flies than the 1-drastic and for the latter to exceed the 2-drastic ones. This fact is brought out in the table by assigning a rank of from 1 to 15 to the mean frequencies from low to high and by summing these ranks in the columns; the orderly increase from 33 through 39 to 48 reflects the trend. This trend fails most obviously in population 1 and, to a lesser degree, in population 5. The exception seen in population 1 is interesting for it is in this population that the greatest proportion of lethals have been exposed to the action of selection for the longest period of time; in the other populations a higher percentage of lethals at any time are of more recent origin.

The different populations also show their characteristic mean values. If the ranks are summed by rows, population 1 has the highest total, 3 is next, and 5 is lowest. These values reflect the differences upon which the relative adaptive values were estimated except that here we are considering only the normal heterozygotes.

Various comparisons of the means of the same and different types of combinations made within and between populations bring to light some interesting and suggestive facts. (*a*) Only in population 6 is there a significant difference between the means of the 2-drastic and the no-drastic combinations. (*b*) 2-drastic combinations of population 5 give fewer wild flies than the same combinations of populations 1, 3 and 6. Similarly, 2-drastics of 6 give fewer wild flies than 2-drastics of 1 or 3. (*c*) 1-drastic combinations of population 5 yield fewer wild flies than similar combinations of population 1, 3 or 6 and, also, this combination in either 6 or 7 yields fewer wild flies than in 1 or 3. (*d*) The normal combinations of populations 5 and 7 gave fewer wild flies than those of 1 and 3. (*e*) The no-drastic combinations of population 5 yield fewer wild flies than 2-drastic or 1-drastic combinations of either 1 or 3 and the no-drastic combinations of population 7 produce fewer wild flies than the 2-drastics of population 1 or the 1-drastic combinations of either 1 or 3.

In spite of the fact that additional data to decrease the errors present in table 2 are desirable, it seems reasonable to conclude that chromosomes within the experimental populations which, when homozygous, reduced viability drastically tended to reduce viability in the heterozygous condition. That selection could operate to counteract this tendency was strongly suggested by the data on population 1. To determine the extent by which drastics lower the viability of heterozygotes, the 2-drastic and 1-drastic combinations of populations 3, 5, 6 and 7 were compared to the normal combinations of these populations. The following are the percentage changes in viability attributable to the 2-drastics: (3) $-2.5\%$, (5) $-1.3\%$, (6) $-5.8\%$ and (7) $-3.2\%$; and to the 1-drastics: (3) $-0.3\%$, (5) $+0.35\%$, (6) $-1.39\%$ and (7) $-0.79\%$. Interestingly, the average change in viability attributable to the 2-drastic and 1-drastic combinations are, respectively, $-3.2\%$ and $-1.4\%$—decreases in nearly a two to one ratio.

These data give us some measure of the effect of lethal and semilethal chromosomes on the adaptive values of the populations. For "standard viability" we can use the $35.16\%$ given in table 1. The simplest comparison is that between the decrease in viability (relative to the standard) exhibited by the no-drastic combinations of populations 3, 5, 6 and 7 and the decrease exhibited by the average "normal" combination without regard to the particular chromosomes involved. The latter can be obtained as weighted averages of the mean viabilities shown in table 2; these averages are: (3) $34.45\%$, (5) $32.00\%$, (6) $33.03\%$ and (7) $33.47\%$. Using population 3 as an example once more, we calculate the difference between $35.16\%$ and $34.53\%$ (the average viability of no-drastic combinations in this population) and divide this difference by $(35.16\% - 34.45\%)$. The result—$89\%$—tells us that $89\%$ of the decrease in viability from the arbitrary standard ($35.16\%$) to the average representative of normal individuals of

population 3 is directly ascribable to the no-drastic heterozygous combinations of that population. Similarly, 99% of the decrease in population 5, 29% of the decrease in population 6 and 82% of the decrease in population 7 can be localized in the no-drastic combinations. The complete elimination of lethal and semilethal chromosomes from these populations, then, would not result in an increase in the average viability to a common level indicated by the standard of 35.16%; the actual increases one would observe after such an elimination would vary from 1 to 71% of the expected increase. (The results of both population 5 and population 6, the two extremes in the above calculations, should be regarded with caution because of the relatively small number of no-drastic combinations available for analysis in those populations. In populations 3 and 7 where larger numbers of combinations were available, the increase observed after elimination of lethal and semilethal chromosomes would seem to be about 15% of that expected on the basis of our "standard.") We conclude then that although nearly all the responsibility for determining the adaptive value of a population lies with the normal individuals of that population, a substantial amount—but far from all—of the effect of these normal individuals is determined by the lethal and semilethal chromosomes carried by them. The individuals from different populations that carry "normal" chromosomes are not identical and the differences between these individuals play extremely large parts in determining the relative adaptive values of the populations. Nor is this latter relation merely a reflection of the effect of these chromosomes when homozygous, for tests of viabilities of homozygotes have consistently indicated a higher average frequency of wild flies in the homozygous test cultures of populatons 3 and 7 than in 1; populations 5 and 6 have given lower frequencies than the other three.

It has already been indicated that individuals heterozygous for lethals and semilethals can, in some populations, have average viabilities lower than individuals from the same populations that carry normal chromosomes. The existence of one population that did not follow the expected pattern indicated that not all lethals and semilethals are equally effective in lowering viability in the heterozygous condition. It may now be asked whether each chromosome tends to have a characteristic effect on the viability of heterozygous individuals regardless of the other chromosome of the combination. It may be mentioned here that flies heterozygous for different second chromosomes do differ in viability; this has been shown in a series of over 100 intercrosses of independently induced second chromosome lethals, in random heterozygotes formed in the routine tests of the experimental populations, and in a special series of more than 100 heterozygous tests involving only normal chromosomes of high viability when homozygous. The latter series involved counts of nearly 1000 flies per test and, for each population, the probability of getting the observed variability by chance was less than

0.001. Cordeiro[4] has shown a similar heterogeneity among individuals of *D. willistoni* heterozygous for different second chromosomes of that species.

Many instances of divergent viabilities between pairs of heterozygous

TABLE 3

PAIRS OF HETEROZYGOUS COMBINATIONS, INVOLVING COMMON CHROMOSOMES, THAT HAVE WIDELY DIVERGENT VIABILITIES. The NUMERATORS AND DENOMINATIONS GIVE THE VIABILITIES OF THE CHROMOSOMES WHEN HOMOZYGOUS; THE CHROMOSOME COMMON TO BOTH MEMBERS OF EACH PAIR IS ITALICIZED

| | | VIABILITY OF CHROMOSOMES WHEN HOMOZYGOUS, % | VIABILITY OF HETEROZYGOUS COMBINATIONS, % |
|---|---|---|---|
| Pop. 1 | 1 | 33.5/*27.8* | 39.1 |
| | | *27.8*/ 0 | 24.1 |
| | 2 | 33.9/*35.4* | 43.0 |
| | | *35.4*/26.9 | 27.3 |
| | 3 | 35.4/*26.9* | 27.3 |
| | | *26.9*/ 0 | 42.6 |
| | 4 | 20.2/ *0* | 18.3 |
| | | *0* /27.3 | 38.9 |
| | 5 | 13.1/ *1.9* | 21.7 |
| | | *1.9*/30.6 | 38.8 |
| Pop. 3 | 1 | 26.9/*32.8* | 41.2 |
| | | *32.8*/30.8 | 26.8 |
| Pop. 5 | 1 | 0 /*31.4* | 18.2 |
| | | *31.4*/ 0 | 31.0 |
| | 2 | 0 / *0* | 42.7 |
| | | *0* / 0 | 24.4 |
| | 3 | 0 / *6.0* | 16.9 |
| | | *6.0*/29.2 | 34.5 |
| Pop. 6 | 1 | 0 / *0* | 33.5 |
| | | *0* / 7.4 | 16.6 |
| | 2 | 0 / *7.4* | 16.6 |
| | | *7.4*/ 0 | 32.2 |
| | 3 | 35.1/*33.0* | 42.4 |
| | | *33.0*/ 1.9 | 27.3 |
| | 4 | 0 /*35.4* | 41.0 |
| | | *35.4*/ 0 | 18.6 |
| | 5 | 35.4/ *0* | 18.6 |
| | | *0* / 0 | 38.1 |
| Pop. 7 | 1 | 0 /*33.0* | 27.5 |
| | | *33.0*/32.9 | 43.9 |
| | 2 | 34.6/ *0* | 46.1 |
| | | *0* / 0 | 26.3 |

combinations carrying a common second chromosome have been observed in the routine tests. Without considering those pairs in which one combination is lethal or semilethal, there are 2736 tests in the data that can be

compared. With such a large number of observations, numerous pairs of combinations containing a common chromosome should differ in viabilities as a result of chance; over 100 instances with probabilities of 5% or less would be expected through chance error even if the viabilities of all combinations were really identical. In table 3 are listed all observed cases in which the viabilities differed so much that the probability of observing them by chance were they really subsamples of the same statistical population was less than 0.001. One would expect 2 or 3 such instances through chance alone among the 2700 combinations; the total of 16 cases actually observed differs significantly from this expected number. The viability of an individual carrying two second chromosomes, then, depends upon the combination of these two chromosomes; the same chromosome can produce widely divergent viabilities in different combinations. The data of table 3 are presented in fractional form; the two numbers of the fraction indicate the viabilities of the two second chromosomes when each is homozygous. The viability of the heterozygous combination follows the fraction. The chromosome common to the two combinations is italicized. In eight cases (1, 2, 4 and 5 of population 1; 3 of population 5; 3 of population 6; and 1 and 2 of population 7) those combinations with the lowest viabilities involve chromosomes that give the lowest viabilities when homozygous; the reverse is true in four other cases (3 of population 1; 1 of population 3; and 1 and 5 of population 6); and in the remaining four cases the two combinations are composed of chromosomes of similar homozygous viabilities. Cases 4 and 5 of population 6 present an interesting relationship; a chromosome with normal viability when homozygous gave good viability in combination with one lethal but poor with another. However, the second lethal gave good viability when in combination with still another lethal chromosome. A similar situation is seen in cases 1 and 2 of the same population; a semilethal in combination with a lethal gave a heterozygote of poor viability but the lethal in combination with another lethal and the semilethal with still another lethal each produced heterozygotes of good viability. A third example exists in cases 2 and 3 of population 1. It is unfortunate that such widely divergent viabilities were required to give a statistical probability of proper magnitude, still the examples show that a chromosome can produce heterozygous combinations of different viabilities that could not have been accurately predicted from a knowledge of the action of the chromosomes when homozygous.

Case 1 of population 3 is probably one of the few cases in which the divergent viabilites have occurred through chance error. Concurrent with these tests a search was made for "negative heterosis," cases in which heterozygotes had poorer viabilities than either of the two chromosomes involved in the heterozygous combination. No cases of negative heterosis were found; case 1 of population 3 was one of those that were tested further and the original wide divergence was not confirmed.

The unpredictability of viabilities of heterozygous combinations was demonstrated by still another method. Each chromosome was tested in two combinations and, consequently, it was possible to calculate a difference and a standard error of the mean for the viabilities of combinations sharing a common chromosome. Similarly, it was possible to calculate a mean difference and the standard error of the mean for viabilities of heterozygous combinations that did not share a common chromosome. (The original data are tabulated so that adjacent entries represent combinations with a common chromosome. Differences between adjacent entries were compared with differences between non-adjacent entries separated by one culture; symbolically, we compared $(a/b - b/c)$ with $(a/b - c/d)$.) In a series of 301 differences calculated for combinations with a chromosome in common, the mean of differences without regard to sign was $5.01\% \pm 0.23\%$; 312 differences between independent combinations gave $4.74\% \pm 0.20\%$. The knowledge of the viability of a particular chromosome in one combination, then, does not permit a more accurate prediction regarding its viability in a second combination than could be made for a combination of entirely different chromosomes. This situation would apply equally well if all chromosomes of a population were identical for then the distinction between the "same" and "different" chromosomes would be only apparent; the data in table 3 rule out this possibility, however, by showing that these chromosomes—and their combinations—are not identical.

Some of the complex interactions involved in the establishment of the adaptive value of a population through the action of selection on the genetic structure are now apparent. The manifestation of lethal or semilethal chromosomes in individuals of markedly poor viability has a minor influence on the population as a whole. These chromosomes exert a greater effect on the population through their action in "normal" individuals; within some populations, at least, individuals carrying lethal or semilethal chromosomes have demonstrably poorer viabilities, on the average than those that carry chromosomes giving higher viabilities when homozygous. It was also demonstrated, however, that the viabilities of individuals carrying only "normal" chromosomes varied from population to population and that these individuals were frequently the most influential determiners of the estimated adaptive values. It appears that the viability of a combination of two chromosomes is characteristic of the combination, not of either one of the two chromosomes. The action of selection, then, cannot be to retain this or that chromosome in a population because of its characteristics when homozygous or because of its characteristics when in any one of several possible combinations; rather, selection must act to retain within a population those chromosomes whose complex of combinations and recombinations gives, on the average, the highest adaptive value.

Dobzhansky[6] showed that the three cytologically distinguishable third

chromosomes (ST, AR and TL) of *D. pseudoobscura* were retained in a population only through the superiority of AR/TL heterozygotes and ST/AR heterozygotes. ST/TL heterozygotes were inferior to ST/ST homozygotes and, if it were not for the other, heterotic combinations, TL would have been eliminated by selection. The frequency of each chromosome finally established by selection was that which gave the highest average adaptive value to the entire complex. These three variables, then, lead to a fairly complicated situation; all the subtle interactions of the many individual AR, ST and TL chromosomes with one another is staggering.

It seems impossible to avoid the conclusion that an isolated population, within surprisingly few generations after its inception, achieves as a result of selection a characteristic and unique integration of its genetic pool. It develops a genetic personality. In the present studies, the differing histories of the populations have resulted in the establishment of gene pools identifiable by routine tests on frequencies of lethals and semilethals, average viabilities of non-lethal, non-semilethal chromosomes when homozygous, average viabilities of random combinations of second chromosomes, and to a limited extent, frequency of lethal and semilethal heterozygous combinations.

*Summary.*—An analysis of viabilities of individuals heterozygous for chromosomes sampled from a number of experimental populations of *D. melanogaster* has made it possible to identify the determinants of the adaptive values of the populations. Individuals of drastically reduced viabilities are so rare in populations that they have little effect in determining the adaptive values. Of the normal individuals, those carrying lethal or semilethal chromosomes have, in some populations, lowered average viabilities (1–3% lower than individuals carrying "normal" chromosomes). In these same populations, however, the individuals carrying two normal second chromosomes have viabilities that account for anywhere from 29 to 99% of the reductions in adaptive values.

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# ON THE THEORY OF DYNAMIC PROGRAMMING

BY RICHARD BELLMAN

THE RAND CORPORATION, SANTA MONICA, CALIFORNIA

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*1. Introduction.*—We are interested in a class of mathematical problems which arise in connection with situations which require that a bounded or unbounded sequence of operations be performed for the purpose of achieving a desired result. Particularly important are the cases where each operation gives rise to a stochastic event, the result of which is applied to the determination of subsequent operations.

Two fundamental problems encountered in situations of this type, in some sense duals of each other, are those of maximizing the yield obtained in a given time, or of minimizing the time or cost required to accomplish a certain task.

In many cases, the problem of determining an optimal sequence of operations may be reduced to that of determining an optimal first operation. The general class of functional equations generated by problems of this nature has the form

$$f(p) = \begin{Bmatrix} \text{min.} \\ \text{max.} \\ k \end{Bmatrix} (T_k(f)), \tag{1.1}$$

where $T_k$ is an operator. In many cases of interest, the operator has the form

$$T_k(f) = g_k(p) + h_k(p)f(S_k p), \tag{1.2}$$

where $S_k$ is a point transformation.

We shall first present some existence and uniqueness theorems pertaining to the solutions of (1.1), and then present explicit solutions of some simple functional equations of the form of (1.1).

As simple examples of problems which give rise to functional equations of this form, we mention the following:

1. We are given the fact that one of $N$ boxes contains a ball, with probability $p_k$ that the ball is in the $k$th box. Let $q_k$ be the probability that on examining the $k$th box we are unable to examine its contents, and $t_k$ be the time consumed in one examination. What procedure minimizes the expected time required to locate the box containing the ball, and what procedure minimizes the expected time required to obtain the ball?

2. We are given a quantity $x > 0$ which may be divided into two parts, $y$ and $x - y$. From $y$ we obtain a return of $g(y)$ and from $x - y$ a return of $h(x - y)$. In so doing we are left with a new quantity $ay + b(x - y)$, $0 < a, b < 1$, with which to continue the process. How does one pro-

ceed in order to maximize the total return obtained in a finite, or unbounded, number of stages?

The theory of dynamic programming is intimately related to the theory of sequential analysis due to Wald.[3] Two papers by Arrow, Blackwell and Girshick,[1] and Arrow, Harris and Marschak[2] also treat problems of similar type.

*2. Existence and Uniqueness Theorems.*

THEOREM 1. *Consider the equation*†

$$f(p) = \max_{1 \leq k \leq n} (g_k(p) + h_k(p) f(S_k p)), \qquad p \in R, \tag{2.1}$$

*where we assume that*

$$\begin{aligned} &(a) \quad \text{If } p \in R, \text{ a region of } n\text{-dimensional space, then } S_k p \in R. \\ &(b) \quad |g_k(p)| \leq c_1 \qquad \text{for } p \in R, \\ &(c) \quad |h_k(p)| \leq c_2 < 1 \qquad \text{for } p \in R, \\ &(d) \quad g_k(p),\ h_k(p) \geq 0 \qquad \text{for } p \in R. \end{aligned} \tag{2.2}$$

*Under these assumptions there is a unique bounded solution to (2.1).*

THEOREM 2. *Consider the equation*

$$f(x) = \max_R [a(x_1, x_2, \ldots, x_N) + f(b(x_1, x_2, \ldots, x_N))], \tag{2.3}$$

*where $R = R(x)$ is defined by $x_k \geq 0, \sum_{k=1}^{N} x_k = x$.*

*If*

$$\begin{aligned} &(a) \quad a(x_1, x_2, \ldots, x_N) \text{ is continuous over } R(x) \text{ for } 0 \leq x \leq x_0, \text{ non-} \\ &\qquad \text{negative, and } a(0, 0, \ldots, 0) = 0, \\ &(b) \quad b(x_1, x_2, \ldots, x_N) \text{ is continuous and non-negative over } R, \\ &(c) \quad b(x_1, x_2, \ldots, x_N) \leq cx,\ 0 < c < 1, \text{ in } R(x), \\ &(d) \quad \sum_{l=0}^{\infty} h(c^l x_0) < \infty, \text{ where } h(x) = \max_R a(x_1, x_2, \ldots, x_N), \end{aligned} \tag{2.4}$$

*there is a unique continuous solution to (2.3) for which $f(0) = 0$ for $0 \leq x \leq x_0$.*

THEOREM 3. *Consider the equation*

$$\begin{aligned} f(p) &= \min \begin{Bmatrix} 1 + \sum_{k=0}^{N} p_k f(x_k), \\ 1 + f(S_l p) \end{Bmatrix}, \qquad p \neq x_0, \\ f(x_0) &= 0, \end{aligned} \tag{2.5}$$

*where $l = 1, 2, \ldots, M$, and*

$$p = \begin{pmatrix} p_0 \\ p_1 \\ \vdots \\ p_N \end{pmatrix}, \quad S_l p = \begin{pmatrix} p_{0l} \\ p_{1l} \\ \vdots \\ p_{Nl} \end{pmatrix}, \quad x_k = \begin{pmatrix} 0 \\ \vdots \\ 1 \\ \vdots \\ 0 \end{pmatrix}, \tag{2.6}$$

*the 1 occurring in the kth place. Each p and $S_l p$ is a probability vector, $p_k \geq 0$, $\sum_{k=0}^{N} p_k = 1$, and $f(p)$ is a scalar function of p.*

*If for each l it is true that*

$$\sum_{k=1}^{N} p_{kl} \leq c_1 \sum_{k=1}^{N} p_k, \quad 0 < c_1 < 1, \tag{2.7}$$

*there is a unique bounded positive solution to (2.5).*

The proof in all three cases employs the method of successive approximations. The equation in (2.5) occurs in connection with problems similar to problem 1 above.

*3. Solutions of Some Particular Functional Equations.*—In this section we indicate the solution of some simple cases of the general equations discussed above.

THEOREM 4. *The solution of*

$$f(x, y) = \max. \begin{bmatrix} p_1[r_1 x + f(s_1 x, y)] \\ p_2[r_2 y + f(x, s_2 y)] \end{bmatrix}, \quad x, y \geq 0 \tag{3.1}$$

*where $0 < p_1, p_2, s_1, s_2 < 1$, $r_1, r_2 > 0$, is given by*

$$\begin{aligned} f(x, y) &= p_1[r_1 x + f(s_1 x, y)] \quad \text{for} \quad \frac{p_1 r_1 x}{1 - p_1} \geq \frac{p_2 r_2 y}{1 - p_2}, \\ &= p_2[r_2 y + f(x, s_2 y)] \quad \text{for} \quad \frac{p_1 r_1 x}{1 - p_1} \leq \frac{p_2 r_2 y}{1 - p_2}. \end{aligned} \tag{3.2}$$

*If $s_1^m = s_2^n$, $f(x, y)$ is piecewise linear.*

This result may be extended in many ways.

THEOREM 5. *The solution of*

$$f(x) = \max_{0 \leq y \leq x} [g(y) + h(x - y) + f(ay + b(x - y)], \tag{3.3}$$

*where $0 < a, b < 1$, may be reduced to that of*

$$f(x) = \max. [g(x) + f(ax), h(x) + f(bx)], \tag{3.4}$$

*in $0 \leq x \leq x_0$, if g and h are monotonically increasing functions such that $g(0) = h(0) = 0$, $g'', h'' \geq 0$ in $[0, x_0]$.*

If $g'', h'' \leq 0$ the situation is much more complicated, and no such simple result such as (3.4) holds in general. The solution of (3.4) may be obtained explicitly and is similar in structure to that of (3.1) above. This functional equation arises from problem 2.

THEOREM 6. *The solution of*

$$f(p_1, p_2, \ldots, p_N) = \min_k \left[ \frac{t_k}{1 - q_k} + (1 - p_k) f\left( \frac{p_1}{1 - p_k}, \ldots, 0, \ldots, \frac{p_N}{1 - p_k} \right) \right] \tag{3.5}$$

*the zero occurring in the kth place, where*

$$f(0, \ldots, 0, p_k, \ldots, 0) = \frac{t_k}{1 - q_k}, \tag{3.6}$$

*for* $p_k > 0$, $k = 1, 2, \ldots, N$, *is given by*

$$f(p_1, p_2, \ldots, p_N) = \frac{t_k}{1 - q_k} + (1 - p_k) f \times \left( \frac{p_1}{1 - p_k}, \ldots, 0, \ldots, \frac{p_N}{1 - p_k} \right) \tag{3.7}$$

*if k is the index for which* $p_i(1 - q_i)/t_i$ *is a maximum.*

This is the solution to problem 1 above in the case where we wish to obtain the ball. If we want merely to locate the ball the solution is more complicated. In this case we either examine the box for which $p_k(1 - q_k)/t_k$ is a maximum first, or we never examine it.

THEOREM 7. *The solution of*‡

$$f(x) = 1 + \min \begin{Bmatrix} xf(1) \\ f(ax) \end{Bmatrix}, \qquad 1 \geq x > 0, \; 0 < a < 1, \tag{3.8}$$
$$f(0) = 0,$$

*is*

$$\begin{aligned} f(x) &= 1 + xf(1), \qquad x \leq x_0 \\ &= 1 + f(ax), \qquad x \geq x_0, \end{aligned} \tag{3.9}$$

*where* $x_0 = (1 - a^k)/(k + 1)(1 - a)$, *and k is the integer at which* $(y + 1)/(1 - a^y)$ *is a minimum for* $y = 1, 2, \ldots$.

Detailed proofs and further results will appear in another publication.

† Results on the existence of solutions of (2.1) were obtained by S. Karlin and H. N. Shapiro in an unpublished paper.

‡ The solution of (3.1) was obtained in conjunction with M. Shiffman, while that of (3.8) was obtained in conjunction with D. Blackwell.

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# THE ITERATION OF THE STEENROD SQUARES IN ALGEBRAIC TOPOLOGY

BY JOSÉ ADEM

PRINCETON UNIVERSITY

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In this work it is proved that the Steenrod squares on cohomology classes satisfy certain identities. These identities are applied to solve particular homotopy problems. Full details will appear elsewhere.

1. Let $H^q(K)$ be the $q$th cohomology group of a complex $K$ with the integers mod 2 as coefficient group. The Steenrod squares,[1] denoted by $\mathrm{Sq}^i$, $(i = 0, 1, \ldots)$, are invariant homomorphisms

$$\mathrm{Sq}^i\colon H^q(K) \to H^{q+i}(K),$$

defined for any complex $K$, satisfying, among several others, the following properties: for any map $f$ of one complex into another, $f^*\mathrm{Sq}^i = \mathrm{Sq}^i f^*$; $\mathrm{Sq}^0 =$ identity; $\mathrm{Sq}^q u = u \smile u$, (mod 2), if $q = \dim u$.

An *iterated* square is a composition of two or more of the squares, e.g., $\mathrm{Sq}^i\mathrm{Sq}^j\mathrm{Sq}^k$. The following relations (mod 2) on iterated squares are well known

$$\mathrm{Sq}^1\mathrm{Sq}^k = \begin{cases} \mathrm{Sq}^{k+1} & \text{when } k \text{ is even,} \\ 0 & \text{when } k \text{ is odd.} \end{cases}$$

A principal result of this paper is a system of additional relations as follows.

THEOREM 1.1. *For all $s > t$, the squares satisfy the mod 2 relations*

$$\mathrm{Sq}^{2t}\mathrm{Sq}^s = \sum_{j=0}^{t} \binom{s - t + j - 1}{2j} \mathrm{Sq}^{t+s+j}\,\mathrm{Sq}^{t-j},$$

where $\binom{k}{j}$ *is the mod 2 value of the binomial coefficient, with the convention:* $\binom{k}{j} = 0$, *if* $j > k$.

From these relations we obtain

$$\mathrm{Sq}^{2^n(2r+1)} = \mathrm{Sq}^{2^n}\mathrm{Sq}^{2^{n+1}r} + \sum_{p=0}^{n-1} \mathrm{Sq}^{2^n(2r+1)-2^p}\mathrm{Sq}^{2^p}. \tag{1.2}$$

As special cases of 1.2, we have

$$\mathrm{Sq}^{2^n}\mathrm{Sq}^{2^m} = \mathrm{Sq}^{2^m+2^n} + \sum_{p=0}^{n-1} \mathrm{Sq}^{2^m+2^n-2^p}\mathrm{Sq}^{2^p}, \qquad (m > n), \tag{1.3}$$

$$\text{(1.4)} \qquad \mathrm{Sq}^{2^n}\mathrm{Sq}^{2^n} = \sum_{p=0}^{n-1} \mathrm{Sq}^{2^{n+1}-2^p}\mathrm{Sq}^{2^p}.$$

A direct consequence of 1.2 is

THEOREM 1.5. *The squares of the type* $\mathrm{Sq}^{2^p}$ *with* $p = 0, 1, \ldots,$ *form a base for the squaring operations. Precisely, the relations 1.2 can be used to write any square as a sum of iterated squares of exponents* $2^p$*; e.g.,* $\mathrm{Sq}^6 = \mathrm{Sq}^2\mathrm{Sq}^4 + \mathrm{Sq}^1\mathrm{Sq}^4\mathrm{Sq}^1$.

*Remark:* Using a different approach by means of Eilenberg-MacLane complexes, Serre[2] has proved that the iterated squares of the type $\mathrm{Sq}^{i_1}\mathrm{Sq}^{i_2}\ldots\mathrm{Sq}^{i_k}$, with $i_1 \geq 2i_2, \ldots, i_{k-1} \geq 2i_k$, form a base for the iterated squares. Using the relations 1.1, a new proof of this fact can be given. It has the advantage that explicit formulae are obtained for expressing an arbitrary iterated square in terms of this base.

2. Let $u$ be a cohomology class of a complex $K$, with coefficients in a group $G$. Using 1.2 we can write the following expression for the self-product of $u$.

If $q = \dim u = 2^n(2r+1)$, $r > 0$, then

$$\text{(2.1)} \qquad u \smile u = \mathrm{Sq}^{2^n}\mathrm{Sq}^{q-2^n}u + \sum_{p=0}^{n-1} \mathrm{Sq}^{q-2^p}\mathrm{Sq}^{2^p}u, \qquad (\mathrm{mod}\ 2).$$

If $q+1 = \dim u = 2^n(2r+1)+1$, $r > 0$, $n > 0$, then

$$\text{(2.2)} \qquad u \smile u = \delta^*\left[\mathrm{Sq}^{2^n}\mathrm{Sq}^{q-2^n}u + \sum_{p=0}^{n-1} \mathrm{Sq}^{q-2^p}\mathrm{Sq}^{2^p}u\right],$$

where $\delta^*$ is the coboundary homomorphism of the coefficient sequence $0 \to G \to G \to G/2G \to 0$[3]. In this last case we suppose $G$ has no elements of order two.

These formulae impose strong restrictions on self-products in the cohomology ring of a complex. As an example, 2.2 implies: *A complex* $K$ *cannot exist with an integer 7-class* $u$*, such that* $u \smile u \neq 0$*, and* $H^{11}(K;\ \mathrm{mod}\ 2) = 0$.

3. We call an even-dimensional manifold $M$, of *type* $\alpha$, if it is connected, orientable, and its middle Betti number is 1. That is, $\dim M = 2k$ and $R_0 = R_k = R_{2k} = 1$. As is well known, a manifold of type $\alpha$ with $k$ odd cannot exist. This is a direct consequence of Poincaré duality and the anticommutativity of the cup-product. By using Poincaré duality and formula 2.1 the following further restrictions can be given on the homology structure of manifolds.

THEOREM 3.1. *Let* $\dim M = 2k = 2^{n+1}(2r+1)$, $r > 0$. *Then there does not exist a manifold* $M$ *of type* $\alpha$ *such that* $H^{2^n}(M;\ \mathrm{mod}\ 2) = 0$ *and* $H^{k-2^p}(M;\ \mathrm{mod}\ 2) = 0$, *for* $p = 1, \ldots, n-1$.

A direct consequence of 3.1 is the following.

COROLLARY 3.2. *If there exists a 2k-dimensional manifold, without torsion, and with Poincaré polynomial* $1 + x^k + x^{2k}$, *then k is a power of two.*

*Remark:* Theorem 3.1 solves partially a problem, considered by Hirsch[4] and Bassi[5], about the existence of a 12-dimensional manifold with Poincaré polynomial $1 + x^6 + x^{12}$. It follows from 3.1 that such a manifold $M$ does not exist with $H^2(M;\ \text{mod } 2) = 0$.

4. Given a map $f\colon\ S^{p+q-1} \to S^q$ (of a $(p + q - 1)$-sphere into a $q$-sphere), let $K$ be the complex obtained by adjoining to $S^q$ a $(p + q)$-cell by means of $f$. The map is essential if $\mathrm{Sq}^p\colon\ H^q(K) \to H^{p+q}(K)$ is different from zero. Since the intermediate cohomology groups of $K$ are zero, it follows from 1.5 that $\mathrm{Sq}^p$ will be zero if $p$ is not a power of two.

Hopf has constructed[6] essential maps $f\colon\ S^{2n-1} \to S^n$ for all even $n$. He assigned to any such map an integer called the Hopf invariant $H(f)$ (an invariant of the homotopy class which is zero for inessential maps). For each even $n$, he exhibited maps $f$ for any even $H(f)$. For $n = 1, 2, 4, 8$, he showed that any integer could be an $H(f)$. Recently G. Whitehead[7] proved that $H(f)$ is even for any $f$ when $n = 4k + 2$ $(k \geq 1)$. In the problem of deciding for which $n$'s a map of Hopf invariant 1 exists, we will exclude many values of $n$ with the following argument. The mod 2 value of $H(f)$ is 0 or 1 according as $\mathrm{Sq}^n\colon\ H^n(K) \to H^{2n}(K)$ is 0 or not. Thus the result of the preceding paragraph proves the following.

THEOREM 4.1. *The Hopf invariant* $H(f)$ *of a map* $f\colon S^{2n-1} \to S^n$ *must be even if n is not a power of 2.*

The problem remains open of deciding whether or not there exists for each power of 2 a map of Hopf invariant 1.

It is well known that (4.1) has several implications; we will mention only the following.

COROLLARY 4.2. *A representation of* $S^m$ *as a sphere bundle over* $S^n$ *is possible only if* $m = 2n - 1$ *and n is a power of 2.*

This corollary holds for an arbitrary group of the bundle. For the rotation group $R_n$ as group of the bundle, it was proved recently[8] by N. E. Steenrod and J. H. C. Whitehead.

COROLLARY 4.3. *A map* $f\colon\ S^n \times S^n \to S^n$ *of type* $(1, 1)$ *can exist only if* $n + 1$ *is a power of 2.*

Hopf[9] and Behrend[10] studied the question of the existence of real division algebras in the euclidean $n$-space (i.e., a bilinear multiplication with a two-sided unity and without 0-divisors). They showed that this does not exist when $n$ is not a power of 2. The preceding result enables us to improve on this result by the elimination of the condition of bilinearity.

5. By extending the process of adjoining cells to spheres, and by means of formulae 1.3 and 1.4, we prove the following general theorem about the compositions of suspensions of maps with Hopf invariant 1.

THEOREM 5.1. *Let $f: S^{2m-1} \to S^m$ and $g: S^{2n-1} \to S^n$ be maps with Hopf invariant 1. Suppose $m \geq n$. Let $g'$ be the $(2m - 1 - n)$-fold suspension of $g$. Then the composition $fg'$ is always essential; moreover*

*(1) if $m = n$, the $p$-fold suspension of this composed map is essential for all $p$,*

*(2) if $m > n$, the $p$-fold suspension of this composed map is essential for all $p < n$. All the essential elements constructed in this way are not divisible by 2.*

If, in the above, we take $f = g$ to be the map $S^3 \to S^2$ of Hopf invariant 1, then we obtain a new proof that $\pi_{n+2}(S^n) \neq 0$ for $n \geq 2$. Similarly, if $f = g$ is the map $S^7 \to S^4$ (respectively, $S^{15} \to S^8$) of Hopf invariant 1, we find that $\pi_{n+6}(S^n) \neq 0$ for $n \geq 4$ (respectively, $\pi_{n+14}(S^n) \neq 0$ for $n \geq 8$).

6. We shall indicate how our relations on iterated squares can be used to construct some cohomology operations of the *second kind*. Let $Z$ and $Z_2$ be the group of integers and integers mod 2, respectively, and suppose $\mathrm{Sq}^2$: $H^q(K; Z) \to H^{q+2}(K; Z_2)$ defined with the natural pairing. Define $N^q(K) \subset H^q(K; Z)$ to be the kernel of $\mathrm{Sq}^2$. We shall construct an operation of the second kind

$$\Phi: N^q(K) \to H^{q+3}(K; Z_2)/\mathrm{Sq}^2 H^{q+1}(K, Z). \tag{6.1}$$

First, consider $u \in H^q(K; Z)$ with $q \geq 2$, and let $u_1$ be a representative of $u$. With $n = 1$ in 1.4 we have the relation $\mathrm{Sq}^2\mathrm{Sq}^2 u + \mathrm{Sq}^3\mathrm{Sq}^1 u = 0$. The proof of our relations on iterated squares is by means of a cochain construction. For this particular relation we construct cochain mappings

$$E_j: C^t(K^4) \to C^{t-j}(K),$$

where $K^4 = K \times K \times K \times K$, such that mod 2

$$(u_1 \smile_i u_1) \smile_{i+2} (u_1 \smile_i u_1) + (u_1 \smile_{i+1} u_1) \smile_i (u_1 \smile_{i+1} u_1) = \delta E_{3i+3}(u_1^4),$$

where $\smile_k$ is the cup-$k$ product[1] and $i = q - 2$. If $u$ is a class with integer coefficients, then $\mathrm{Sq}^1 u = 0$, therefore $\mathrm{Sq}^2\mathrm{Sq}^2 u = 0$. In this case a mod 2 cochain expression for this last relation is given by

$$(u_1 \smile_i u_1) \smile_{i+2} (u_1 \smile_i u_1) = \delta[E_{3i+3}(u_1^4) + \eta(u_1) \smile_{i-1} \eta(u_1) + \eta(u_1) \smile_i \delta\eta(u_1)], \tag{6.2}$$

where,

$$\eta(u_1) = {}^1/_2[u_1 \smile_{i+2} u_1 + u_1].$$

Now, suppose that $\mathrm{Sq}^2 u = 0$, that is $u \in N^q(K)$. Then for some cochain $b$ we have $u_1 \smile_i u_1 = \delta b$. It follows mod 2 that,

$$(u_1 \smile_i u_1) \smile_{i+2} (u_1 \smile_i u_1) = \delta[b \smile_{i+1} b + b \smile_{i+2} \delta b]. \tag{6.3}$$

Therefore, in 6.2 and 6.3 we have the same expression being a coboundary for two different reasons. It follows from a general principle, already used by Steenrod on defining functional operations, that both expressions together will give rise to a new cohomology operation. By setting

$$w_1 = b \smile_{i+1} b + b \smile_{i+2} \delta b + E_{3i+3}(u_1^4) + \eta(u_1) \smile_{i-1} \eta(u_1) + \eta(u_1) \smile_i \delta\eta(u_1),$$

it follows from 6.2 and 6.3 that $w_1$ is a mod 2 cocycle and we define our operation of 6.1 by

$$\Phi u = \{w_1\} + \mathrm{Sq}^2 H^{q+1}(K;\ Z).$$

The operation $\Phi$ can be proved to be a well-defined operation, independent of all the arbitrariness that may occur in our construction. Some of its properties are: (1) $f^*\Phi = \Phi f^*$, for any map $f$ of one complex into another, therefore, it is an invariant operation; (2) the definition of $\Phi$ extends to the relative case and there $\Phi$ commutes with the coboundary operator; (3) $\Phi$ is a homomorphism; (4) $\Phi$ is an effectively computable operation. The fact that this operation may not be trivial is shown by the following.

According to the pattern introduced by Steenrod,[11] with the operation $\Phi$ and a map $f$ we can define the functional operation $\Phi_f$.

THEOREM 6.4. *Let $f$: $S^{n+2} \to S^n$ be a map of an $(n+2)$-sphere into an $n$-sphere with $n \geq 2$. The functional operation*

$$\Phi_f:\ H^n(S^n;\ Z_2) \to H^{n+2}(S^{n+2};\ Z_2),$$

*is non-trivial if and only if the map $f$ is essential.*

In the above theorem a cochain expression can be given for $\Phi_f$ and that provides an effective method for deciding the homotopy class of a given simplicial map $f$: $S^{n+2} \to S^n$.

Let $K$ be the cell complex constructed by adjoining a cell $E^{n+3}$ to $S^n$, by means of the map $f$: $S^{n+2} \to S^n$. It follows from 6.4 that $\Phi$: $H^n(K;\ Z_2) \to H^{n+3}(K;\ Z_2)$ will map the generator of $H^n(K;\ Z_2)$ onto the generator of $H^{n+3}(K;\ Z_2)$, if and only if the given map $f$ is essential.

The main application of the new operation is the computation of the *third obstruction*.

THEOREM 6.5. *Let $f$: $K^n \to S^n$ be a map from the $n$-skeleton of a complex $K$ into an $n$-sphere. Let $s^n$ be a generator of $H^n(S^n)$. Suppose that the first and second obstruction for the extension of this map vanish, i.e., $\delta f^* s^n = 0$ and $\mathrm{Sq}^2 f^* s^n = 0$. Then the third obstruction is given by*

$$\Phi f^* s^n \in H^{n+3}(K;\ \pi_{n+2}(S^n))/\mathrm{Sq}^2 H^{n+1}(K;\ \pi_{n+1}(S^n)).$$

In this last theorem the pairings for the different coefficient groups are the natural ones. By following the general pattern given by Steenrod,[1] the maps of an $(n+2)$-complex into an $n$-sphere can be classified. In particu-

lar, we can compute the cohomotopy groups $\pi^n(K^{n+2})$ up to group extensions.

The construction of our operation $\Phi$ corresponds to the relation $Sq^2Sq^2 = Sq^3Sq^1$. By using other relations on iterated squares we can generalize the operation $\Phi$ in an obvious way. In particular, with the relations 1.3 and 1.4 new operations can be constructed, and with them the essential maps obtained in 5.1 can be computed.

7. We shall indicate here the method used in proving our relations on iterated squares.

The procedure devised by Steenrod[12] for constructing the squaring operations is by means of homorphisms of chains of $K$ into those of $K \times K$, which neighbor the diagonal. He uses the cyclic group of order two, operating in $K \times K$ by permutation of the factors. In treating iterated squares we must consider similar homomorphisms from $K$ to $K^4 = K \times K \times K \times K$.

A rough description of our procedure is to parallel the construction of the squares, replacing $K^2$ by $K^4$ and taking as group operating in $K^4$ a group of order four, contained in the dihedral group of order eight. We obtain in this form a new doubly indexed set of invariant operations, $\begin{bmatrix} i \\ k \end{bmatrix}$, each of which is a homomorphism

$$\begin{bmatrix} i \\ k \end{bmatrix}: \ H^q(K) \to H^{4q-i}(K).$$

It is then shown, that $\begin{bmatrix} i \\ k \end{bmatrix}$ coincides with a sum of iterated squares $\sum Sq^rSq^s$. This is achieved by an explicit calculation.

An automorphism of the full dihedral group is used to prove that the $\begin{bmatrix} i \\ k \end{bmatrix}$ contains a symmetry, which is expressed by the relation $\begin{bmatrix} i \\ k \end{bmatrix} = \begin{bmatrix} i \\ i-k \end{bmatrix}$. Thus, for each $i$, $k$ we obtain that one sum of iterated squares equals another. These are the basic identities.

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## *LOCAL TRANSFORMATIONS WITH FIXED POINTS ON COMPLEX SPACES WITH SINGULARITIES*

By S. Bochner and W. T. Martin

Princeton University, Massachusetts Institute of Technology and The Institute for Advanced Study

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We will supplement a recent paper of ours[1] and we first of all describe the nature of the complex space there introduced. Let $S$ be the unit sphere in Euclidean space of $k$ complex variables $z_1, \ldots, z_k$, and over $S$ we consider a system of polynomials in $\zeta$'s,

$$\psi_\nu (z_1, \ldots, z_k, \zeta_1, \ldots, \zeta_\nu) = 0, \ \nu = 1, \ldots, n \tag{1}$$

with coefficients which are analytic in $S$. This defines for us a topological space which will be denoted by $U$. If $R$ is any subset of $S$ then $U(R)$ will denote those points of $U$ whose base points are in $R$. Now there is a function $D(z)$ analytic and not identically zero in $S$ such that for $E$ denoting the points of $S$ where $D$ vanishes the subset $U(E)$ are possible singularities on $U$. However, $U - U(E) = U(S - E)$ shall be a complex analytic coordinate space.

If $m_\nu$ is the degree of $\psi_\nu$ in the variable $\zeta_\nu$ and if $m = m_1 \ldots m_n$ then the system (1) has $m$ sets of solutions

$$\left\{\zeta_1^{(\mu)}(z), \ldots, \zeta_n^{(\mu)}(z)\right\}, \ \mu = 1, \ldots, m. \tag{2}$$

At each point of $S - E$ these sets are assumed distinct, and they shall form $n$ continuous functional elements in a neighborhood of every point of $S - E$.

A function $F(z, \zeta)$ was called *holomorphic* in $U$ if it is defined throughout $U$, is bounded in $U(R)$ for every compact subset $R$ of $S$, and is given in $U(S - E)$ by an expression of the form

$$F(z, \zeta) = \frac{1}{\Delta^2(z)} \sum P_{\lambda_1 \ldots \lambda_n}(z) \zeta_1^{\lambda_1} \ldots \zeta_n^{\lambda_n} \tag{3}$$

where the $P$'s are analytic in $S$. In (3) the summation is taken over the range

$$\lambda_1 = 0, 1, \ldots, m_1 - 1; \ \ldots; \ \lambda_n = 0, 1, \ldots, m_n - 1 \tag{4}$$

and the function $\Delta^2(z)$ is the square of the determinant of the matrix

$$([\zeta_1^{(\mu)}(t)]^{\lambda_1} \ldots [\zeta_n^{(\mu)}(t)]^{\lambda_n}) \tag{5}$$

where $\mu$ ranges over $1, \ldots, m$ and the $\lambda$'s over (4). The function $\Delta^2(z)$ is analytic in $S$, and vanishes in $S$ at most at points of $E$.

Such a space being given, we now say that a mapping $T$ of $U$ into $U$ is a *continuous holomorphic mapping* if it is given by

$$T: \quad \begin{aligned} w_j &= F_j(z, \zeta), \ j = 1, \ldots, k \\ \zeta_\nu &= G_\nu(z, \zeta), \ \nu = 1, \ldots, n \end{aligned} \tag{6}$$

where the $F_j$ and $G_\nu$ are continuous and holomorphic in $U$, and the $G$'s are related to the $F$'s in such a manner that the points $(w, \zeta) \in U$. If $T$ and $T'$ are two such mappings, and if $TU$ is not entirely in $U(E)$ then we shall show that the iterated transformation $T'T$ is also a continuous holomorphic mapping of $U$ into $U$. This result is an immediate consequence of the following theorem which we will establish.

THEOREM 1. *Let (6) be a continuous holomorphic mapping of $U$ into $U$ such that $TU - U(E)$ is not empty. Let $H(z, \zeta)$ be continuous and holomorphic in $U$. Then*

$$H[F_1(z, \zeta), \ldots, F_k(z, \zeta); \ G_1(z, \zeta), \ldots, G_n(z, \zeta)] \tag{7}$$

*is continuous and holomorphic in $U$.*

*Proof:* Clearly the function (7) is continuous in $U$. In order to see that it is holomorphic we first develop $H(z, \zeta)$ as in the right-hand side of (3), and make the transformation (6). This leads to a representation of (7) as the quotient of two expressions

$$\sum P_{\lambda_1 \ldots \lambda_n}[F_1(z, \zeta), \ldots, F_k(z, \zeta)][G_1(z, \zeta)]^{\lambda_1} \ldots [G_n(z, \zeta)]^{\lambda_n} \tag{8}$$

and

$$\Delta^2[F_1(z, \zeta), \ldots, F_k(z, \zeta)], \tag{9}$$

valid in that part of $U$ for which the expression (9) is different from zero. Since $TU - U(E)$ is assumed non-empty, and since $\Delta^2(z)$ vanishes in $S$ only over points of $E$, the function (9) is not identically zero.

The remainder of the proof of Theorem 1 will follow from three lemmas, of which Lemma 2 will be preliminary to Lemma 3.

LEMMA 1. *Let $F_1(z, \zeta), \ldots, F_s(z, \zeta)$ be $s$ functions each continuous and holomorphic in $U$, and let $\Phi(w_1, \ldots, w_s)$ be analytic in a region $B$. If the values*

$$w_\sigma = F_\sigma(z, \zeta), \ \sigma = 1, \ldots, s, \ (z, \zeta) \in U$$

*lie in B, then the function*

$$F(z, \zeta) = \Phi[F_1(z, \zeta), \ldots, F_s(z, \zeta)] \tag{10}$$

*is continuous and holomorphic in U.*

LEMMA 2. *Let $I(z, \zeta)$ be holomorphic in U, let $\Omega(z)$ be analytic and not identically zero in S, and let $F(z, \zeta)$ be continuous in U. If*

$$F(z, \zeta) = \frac{I(z, \zeta)}{\Omega(z)} \text{ in } U(S - E - E_\Omega) \tag{11}$$

*where $E_\Omega$ is the subset of S where $\Omega$ vanishes, then F is holomorphic in U.*

LEMMA 3. *If $H_1(z, \zeta)$ and $H_2(z, \zeta)$ are holomorphic in U, if $H_2(z, \zeta)$ is continuous and $\not\equiv 0$ in U, if $F(z, \zeta)$ is continuous in U and if*

$$F(z, \zeta) = \frac{H_1(z, \zeta)}{H_2(z, \zeta)} \tag{12}$$

*in the part of $U(S - E)$ where $H_2 \neq 0$, then F is holomorphic in U.*

*Proof of Lemma 1:* The function (10) is clearly continuous. In order to see that it is holomorphic we first fix $\lambda_1, \ldots, \lambda_n$ in (4) and denote by $\Delta'_{\lambda_1 \ldots \lambda_n}(z)$ the matrix obtained from the matrix (5) by replacing the elements of the column, one element of which is displayed in (5), by $f_1(z), \ldots, f_m(z)$, where $f_\mu(z) = F(z, \zeta_1^{(\mu)}(z), \ldots, \zeta_n^{(\mu)}(z))$. We denote by $\Delta_{\lambda_1 \ldots \lambda_n}(z)$ the determinant of $\Delta'_{\lambda_1 \ldots \lambda_n}(z)$ and define

$$P_{\lambda_1 \ldots \lambda_n}(z) = \Delta_{\lambda_1 \ldots \lambda_n}(z)\Delta(z). \tag{13}$$

The functions $P_{\lambda_1 \ldots \lambda_n}$ so defined arise from symmetric functions in $(\zeta_1^{(\mu)}, \ldots, \zeta_n^{(\mu)})$, $\mu = 1, \ldots, m$, and are continuous in $S$ and analytic in $S - E$. Since the set $E$ is a removable exceptional set, it follows that each of the $P$'s is analytic in all of $S$.

Next consider the $(m + 1) \times (m + 1)$-matrix

$$\begin{pmatrix} F(z, \zeta) & \ldots & \{\zeta_1^{\lambda_1} \ldots \zeta_n^{\lambda_n}\} & \ldots \\ \cdots & \cdots & \cdots & \cdots \\ f_\mu(z) & \ldots & \{[\zeta_1^{(\mu)}(z)]^{\lambda_1} \ldots [\zeta_n^{(\mu)}(z)]^{\lambda_n}\} & \ldots \\ \cdots & \cdots & \cdots & \cdots \end{pmatrix}$$

where we have displayed the first and $(\mu + 1)$-st elements of first column, and the same elements of a typical one of the remaining columns, for the $\lambda$'s in (4). The determinant of this matrix is zero throughout $U$ since it is zero whenever $\zeta_1 = \zeta_1^{(\mu)}(z), \ldots, \zeta_n = \zeta_n^{(\mu)}(z)$ for any $\mu = 1, \ldots, m$. But if we expand the determinant in terms of the elements of the first row, and multiply through by $\Delta(z)$, we obtain

$$\Delta^2(z)F(z, \zeta) = \sum P_{\lambda_1 \ldots \lambda_n}(z)\zeta_1^{\lambda_1} \ldots \zeta_n^{\lambda_n}, \tag{14}$$

the summation being extended over (4). Thus $F$ is holomorphic in $U$.

*Proof of Lemma 2:* As in the proof of Lemma 1, form the functions (13). By the continuity of $F$ and symmetry they are continuous in $S$, and by (11) and symmetry they are analytic in $S - E - E_\Omega$, and hence in $S$. Thus (14) holds, and $F$ is holomorphic.

*Proof of Lemma 3:* By one of the basic assumptions of our space (property (v) of Section 1 in "Complex Spaces"), corresponding to any continuous holomorphic function $H_2(z, \zeta) \not\equiv 0$ in $U$ they are a function $G(z, \zeta)$ strictly holomorphic in $U$ and a function $\Omega(z)$ analytic and not identically zero in $S$ such that $\Delta^2(z)H_2(z, \zeta)G(z, \zeta) \equiv \Omega(z)$ in $U$. It is clear from this that

$$H_2(z, \zeta) \not\equiv 0 \text{ in } U(S - E_\Omega).$$

Hence

$$\frac{1}{H_2(z, \zeta)} = \frac{\Delta^2(z)G(z, \zeta)}{\Omega(z)} \text{ in } U(S - E_\Omega).$$

Thus the function (12) has a representation (11), and by Lemma 2, (12) is holomorphic.

As regards Theorem 1, the first lemma implies that the two functions (8) and (9) are continuous and holomorphic in $U$, and as we have seen, the function (7) is expressible as their quotient in the part of $U$ where the denominator (9) is not zero; thus Lemma 3 completes the proof.

THEOREM 2. *Let $\Gamma$ be an arbitrary compact space, let*

$$F(z, \zeta; \alpha) = \frac{1}{\Delta^2(z)} \sum_{\lambda_1=0}^{m_1-1} \cdots \sum_{\lambda_n=0}^{m_n-1} P_{\lambda_1 \cdots \lambda_n}(z; \alpha)\zeta_1^{\lambda_1} \cdots \zeta_n^{\lambda_n}, \quad (15)$$

*be a family of functions, continuous and holomorphic in $U$ for each $\alpha$ in $\Gamma$, and continuous in $\alpha$ for each $(z, \zeta)$ in $U$. If there are positive numbers $\rho_0 \leq 1$ and $M$ such that*

$$|F(z, \zeta; \alpha)| \leq M, \text{ for } \alpha \in \Gamma \text{ and } (z, \zeta) \in U(\rho_0), \quad (16)$$

*where $U(\rho_0)$ is the subset of $U$ for which $\|z\| < \rho_0$, then the functions $P_\lambda(z; \alpha)$ in (15) have the following properties:*

1°. *For every positive number $\rho < \rho_0$, the $P$'s are uniformly bounded and equi-uniformly continuous in $z$ in $S_\rho$: $\|z\| \leq \rho$;*

2°. *For each $\lambda$ in (4), $P_\lambda(z; \alpha)$ is continuous in $(z, \alpha)$ in $S_\rho \times \Gamma (\rho < \rho_0)$; and*

3°. *The coefficients $p_{s_1 \cdots s_k; \lambda}(\alpha)$ in the power-series expansions*

$$P_\lambda(z; \alpha) = \sum_{s_1=0}^{\infty} \cdots \sum_{s_k=0}^{\infty} p_{s_1 \cdots s_k; \lambda}(\alpha)z_1^{s_1} \cdots z_k^{s_k}$$

*are continuous in $\alpha$.*

*Proof:* The first part of Property 1°, namely the fact that there is a positive number $B\rho$ such that

$$|P_\lambda(z;\alpha)| \leq B_\rho \text{ for } \alpha \in \Gamma,\ z \in S_\rho,\ \lambda \text{ in (4)},$$

is a consequence of Lemma 4.1 of "Complex Spaces." The continuity of $P_\lambda(z;\alpha)$ in $\alpha$ follows from the relationship

$$P_{\lambda_1 \ldots \lambda_n}(z;\alpha) = \Delta_{\lambda_1 \ldots \lambda_n}(z;\alpha)\Delta(z) \text{ in } S,$$

where $\Delta_{\lambda_1 \ldots \lambda_n}(z;\alpha)$ is defined as in (13), and from the continuity of the functions $f_\mu(z;\alpha) = F(z, \zeta_1^{(\mu)}(z), \ldots, \zeta_n^{(\mu)}(z);\alpha)$ in $\alpha$ for fixed $z$ in $S$.

The remaining part of 1° and properties 2° and 3° are consequences of the following lemma.

LEMMA 4. *Let $\Gamma$ be a compact space and let $r_1, \ldots, r_k$ be positive numbers. If*

$$P(z;\alpha) = \sum_{s_1=0}^{\infty} \cdots \sum_{s_k=0}^{\infty} p_{s_1 \ldots s_k}(\alpha) z_1^{s_1} \ldots z_k^{s_k}$$

*is a family of functions continuous in $\alpha$ for each $z$ in the multi-cylinder*

$$C(r):\ |z_1| < r_1, \ldots, |z_k| < r_k$$

*and if there is a positive constant $B$ such that*

$$|p_{s_1 \ldots s_k}(\alpha)| \leq \frac{B}{r_1^{s_1} \ldots r_k^{s_k}},\ \alpha \in \Gamma,\ s_1, \ldots, s_k = 0, 1, 2, \ldots$$

*then*

(i) *for every set of positive numbers $\rho_1, \ldots, \rho_k$ with $\rho_j < r_j$, the functions $P(z;\alpha)$ are equi-uniformly continuous in $z$ in the multi-cylinder*

$$\bar{C}(\rho):\ |z_1| \leq \rho_1, \ldots, |z_k| \leq \rho_k,$$

*and continuous in $(z, \alpha)$ in $C(r) \times \Gamma$; and*

(ii) *the coefficients $p_{s_1 \ldots s_k}(\alpha)$ are continuous in $\alpha$.*

This lemma, which is actually a theorem due to Osgood, is taken as known.

We now take $\Gamma$ to be a compact group, and with each element $\alpha$ in $\Gamma$ we associate a continuous holomorphic 1-1 mapping $T(\alpha)$ of $U$ onto $U$,

$$T(\alpha):\quad \begin{aligned} w_j &= F_j(z, \zeta;\alpha),\ j = 1, \ldots, k \\ \xi_\nu &= G_\nu(z, \zeta;\alpha),\ \nu = 1, \ldots, n \end{aligned} \tag{17}$$

As in Section 1, for any pair $\alpha$, $\beta$ in $\Gamma$ we can define the iterated transformation $T(\alpha)T(\beta)$. We require that this transformation be identical with the transformation $T(\alpha\beta)$, where $\alpha\beta$ is the group product in $\Gamma$.

Under these conditions we will call $\{T(\alpha)\}$ a compact group of continuous holomorphic mappings of $U$ onto $U$.

THEOREM 3. *If (17) is a compact group of continuous holomorphic mappings of $U$ onto $U$, if the functions $F_j(z, \zeta; \alpha)$ are continuous in $\alpha$ for each fixed $(z, \zeta)$ in $U$, and if the functions $F_j$ have the special form*

$$F_j(z, \zeta; \alpha) = z_j + O(\|z\|^{1+\epsilon}), \; j = 1, \ldots, k; \; \alpha \in \Gamma \qquad (18)$$

*for $\|z\|$ sufficiently small, and for some fixed $\epsilon > 0$, then there exists a transformation of $U$ into the base space $S$,*

$$R_1: \; w_j = H_j(z, \zeta) = z_j + O(\|z\|^{1+\epsilon}), \; j = 1, \ldots, k \qquad (19)$$

*defined by $k$ continuous holomorphic functions which has the property that*

$$R_1 T(\alpha) = R_1 \text{ for all } \alpha \text{ in } \Gamma. \qquad (20)$$

*Proof:* Since $T(\alpha)$ maps $U$ onto $U$ we have

$$\left| \sum_{j=1}^{k} F_j(z, \zeta; \alpha) \right|^2 < 1. \qquad (21)$$

By (21), (18) and Schwarz's lemma for holomorphic functions[2] we have

$$\sum_{j=1}^{k} |F_j(z, \zeta; \alpha)|^2 \leq \|z\|^2, \sum_{j=1}^{k} |F_j(z, \zeta; \alpha) - z_j|^2 \leq [2\|z\|^{1+\epsilon}]^2. \qquad (22)$$

Also by (21) the functions $F_j(z, \zeta; \alpha)$ satisfy the hypotheses of Theorem 2 for every $\rho_0 \leq 1$, and hence the related analytic functions $P_{\lambda_1 \ldots \lambda_n, j}(z; \alpha)$ enjoy properties 1°, 2° and 3° of Theorem 2.

If we denote by $T_1(\alpha)$ the transformation

$$T_1(\alpha): \; w_j = F_j(z, \zeta; \alpha) \qquad j = 1, \ldots, k$$

from $U$ into the base space $S$, then we may form the transformation[3-5]

$$R_1 = \int_\Gamma T_1(\alpha) \, d\alpha$$

where $d\alpha$ refers to the Haar measure on $\Gamma$, with total measure unity The transformation $R_1$ is given by

$$w_j = H_j(z, \zeta) = \int_\Gamma F_j(z, \zeta; \alpha) \, d\alpha.$$

Each $H_j$ is continuous in $U$, and on integrating termwise the series developments as in (15) for $F_j$ we see that each $H_j$ is holomorphic in $U$. Also the $H$'s satisfy the same inequalities (22) as the $F$'s.

In order to prove (20) we first note that for any two elements $\alpha, \gamma$ in $\Gamma$

$$T_1(\alpha) T(\gamma) = T_1(\alpha\gamma).$$

Thus

$$R_1T(\gamma) = \int T_1(\alpha)\, d\alpha . T(\gamma) = \int T_1(\alpha)T(\gamma)\, d\alpha = \int T_1(\alpha\gamma)\, d\alpha.$$

By the invariance of Haar measure the latter integral is merely $R_1$.

If $R_1$ were a mapping from $U$ into $U$, rather than from $U$ into $S$, then as in the classical case (20) would imply that each $T(\alpha)$ is the identity.

[1] "Complex Spaces with Singularities," *Ann. Math.* (to appear). In the present note this paper will be referred to as "Complex Spaces."

[2] Schwarz's lemma as given in "Complex Spaces" was formulated for single holomorphic functions and is now being used for several simultaneously.

[3] Cartan, H., *Actualités sci. et ind.*, 198, Paris, 1935.

[4] Bochner, S., *Ann. Math.*, **46**, 372–381 (1945).

[5] "Several Complex Variables," Princeton, 1948.

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## *ON THE SPECTRAL REPRESENTATION OF ORDINARY SELF-ADJOINT DIFFERENTIAL OPERATORS**

BY E. A. CODDINGTON

DEPARTMENT OF MATHEMATICS, MASSACHUSETTS INSTITUTE OF TECHNOLOGY

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*1. Introduction.*—Let the formal ordinary differential operator $L$ be defined by

$$L = p_0(d/dx)^n + p_1(d/dx)^{n-1} + \ldots + p_n,$$

where the $p_k$ are complex-valued functions with $n - k$ continuous derivatives on an open (possibly unbounded) real interval $a < x < b$, and $|p_0(x)| \neq 0$ on $(a, b)$. We assume that $L$ is *formally self-adjoint*, that is, $L$ coincides with its Lagrange adjoint $L^+$ defined by

$$L^+ = (-1)^n(d/dx)^n(\bar{p}_0\cdot) + (-1)^{n-1}(d/dx)^{n-1}(\bar{p}_1\cdot) + \ldots + \bar{p}_n.$$

The object of the present note is to give some results concerning self-adjoint boundary value problems on $(a, b)$ associated with the formal operator $L$.

The case where $n = 2$ and the $p_k$ are real was treated initially by Weyl,[1] and his principal results were given new proofs by Stone,[2] Titchmarsh,[3] Kodaira,[4] Levinson[5] and Yosida.[6] In a subsequent paper[7] Kodaira extended Weyl's results to the case where $n$ is even and the $p_k$ are real. Levitan[8] has considered this case also, and has shown the existence of at least one "spectral matrix" occurring in the Parseval equality. He refers to other papers of M. G. Krein.

The consideration of formal operators $L$ with *complex* coefficients not

only gives rise to new self-adjoint problems, but to new difficulties, since it may not be possible to prescribe *any* boundary conditions which will give a self-adjoint boundary value problem for $L$ on $(a, b)$. An example of this situation is furnished by the operator $L = i(d/dx)$ on $(0, \infty)$. This difficulty cannot arise in the case where all $p_k$ are real.

All of the authors quoted above, except Stone, restricted themselves to boundary value problems with *separated* boundary conditions, that is, a boundary condition at $a$, and another at $b$. That there exist self-adjoint problems with boundary conditions which are not separated is evidenced by examples where *periodic* boundary conditions can occur in the case $n = 2$, $p_k$ real.

*2. The Basic Operators.*—Let $\mathfrak{H}$ denote the Hilbert space of all square summable complex-valued functions on $(a, b)$. We define two operators in $\mathfrak{H}$ associated in a natural way with $L$. Let $\mathfrak{D}$ be the set of all $u \in \mathfrak{H}$ such that $u$ has continuous derivatives up to order $n - 1$ on $(a, b)$, $u^{(n-1)}$ is absolutely continuous on every closed subinterval of $(a, b)$, and $Lu \in \mathfrak{H}$. Define the operator $T$ in $\mathfrak{H}$ by $Tu = Lu$ for $u \in \mathfrak{D}$.

If $a < y < x < b$, and $u$, $v$ are in $\mathfrak{D}$, then Green's formula holds

$$\int_y^x (\bar{v}Lu - u\overline{Lv})dx = [uv](x) - [uv](y),$$

where $[uv](x)$ is a non-singular skew-hermitian semibilinear form in $(u, u', \ldots, u^{(n-1)})$ and $(v, v', \ldots, v^{(n-1)})$. It follows immediately from this formula that if $u, v \in \mathfrak{D}$, then the limits $[uv](a) = \lim [uv](x)$ $(x \to a)$ and $[uv](b) = \lim [uv](x)$ $(x \to b)$ exist. We denote their difference by $< uv >$, that is,

$$< uv > = [uv](b) - [uv](a), \qquad (u, v \in \mathfrak{D}),$$

and define $\mathfrak{D}_0$ to be the set of all $u \in \mathfrak{D}$ such that $< uv > = 0$ for all $v \in \mathfrak{D}$. Define $T_0$ to be the operator with domain $\mathfrak{D}_0$ and $T_0u = Lu$, for $u \in \mathfrak{D}_0$. Clearly $T_0 \subseteq T$.

THEOREM 1. *The operator $T_0$ is closed, symmetric, and its adjoint is $T$.*

This is the $n$th order extension of Theorem 10.11 in the book by Stone.[2] The proof rests upon the classical variation of constants.

Let $\mathfrak{E}(i)$ and $\mathfrak{E}(-i)$ denote the set of all solutions of the equations $Lu = iu$ and $Lu = -iu$, respectively, which are in $\mathfrak{H}$, and put $\omega^+ = \dim \mathfrak{E}(i)$, $\omega^- = \dim \mathfrak{E}(-i)$. The integers $(\omega^+, \omega^-)$ are the deficiency indices of $T_0$, and $T_0$ has self-adjoint extensions if and only if $\omega^+ = \omega^-$. We assume that $\omega^+ = \omega^- = \omega$. Then clearly any self-adjoint extension $H$ of $T_0$ satisfies $T_0 \subseteq H \subseteq T$.

We introduce boundary conditions and show that the set of all self-adjoint extensions of $T_0$ is in a one-to-one correspondence with certain equivalence classes of self-adjoint boundary conditions. A (homogeneous) *boundary condition* is a condition on $u \in \mathfrak{D}$ of the form $< u\alpha_j > = 0$, where

$\alpha$ is a fixed function in $\mathfrak{D}$. The conditions $< u\alpha_j > 0$ $(j = 1, \ldots, m)$ are said to be *linearly independent* if the only set of complex numbers $\gamma_1, \ldots, \gamma_m$ for which $\Sigma\gamma_j < u\alpha_j > = 0$ identically in $u \in \mathfrak{D}$ is $\gamma_1 = \gamma_2 = \ldots = \gamma_m = 0$. A set of $m$ linearly independent boundary conditions $< u\alpha_j > = 0$ $(j = 1, \ldots, m)$ is *self-adjoint* if $< \alpha_j\alpha_k > = 0$ $(j, k = 1, \ldots, m)$.

THEOREM 2. *If $H$ is a self-adjoint extension of $T_0$ with domain $\mathfrak{D}_H$, then there exists a set of $\omega$ self-adjoint boundary conditions*

$$< u\alpha_j > = 0, \qquad (j = 1, \ldots, \omega), \tag{1}$$

*such that $\mathfrak{D}_H$ is the set of all $u \in \mathfrak{D}$ satisfying these conditions. Conversely, if (1) is a set of $\omega$ self-adjoint boundary conditions, there exists a self-adjoint extension $H$ of $T_0$ whose domain is the set of all $u \in \mathfrak{D}$ satisfying (1).*

The proof depends on the von Neumann[9] construction of the self-adjoint extensions of closed symmetric operators using the isometric mappings of $\mathfrak{F}(i)$ onto $\mathfrak{F}(-i)$.

In the special case where $(a, b)$ is a bounded interval, and the functions $p_k$ are continuous on the closed interval $[a, b]$, it is evident that $\omega^+ = \omega^- = n$, and the linearly independent boundary conditions are usually presented in the form

$$\sum_{k=1}^{n} (M_{jk}u^{(k-1)}(a) + N_{jk}u^{(k-1)}(b)) = 0, \qquad (j = 1, \ldots, n), \tag{2}$$

where the $M_{jk}$ and $N_{jk}$ are complex constants. If $M$ and $N$ are the matrices with elements $M_{jk}$ and $N_{jk}$, respectively, then a direct corollary of Theorem 2 is that the conditions (2) are self-adjoint if and only if

$$MB^{-1}(a)M^* = NB^{-1}(b)N^*, \tag{3}$$

where $B(x)$ is the matrix of the semibilinear form $[uv](x)$, and the asterisk denotes the conjugate transposed matrix. A proof of (3) can be given without using the general theory of Hilbert space.[10]

*3. Spectral Considerations.*—Given any self-adjoint extension $H$ of $T_0$ the problem remains to give information concerning the spectrum of $H$. In this note we consider only the case where $T_0$ is self-adjoint, that is, $\omega = 0$, and we denote $T_0$ by $H$. For the case treated by Weyl, this corresponds to the situation where $L$ is of the *limit point* type at $a$ and $b$.

We construct, for every closed bounded subinterval $\delta = [\tilde{a}, \tilde{b}]$ of $(a, b)$, a self-adjoint operator $H_\delta$ which tends, in a certain sense, to the given operator $H$, as $\delta \to (a, b)$. For each such $\delta$, consider an arbitrary, but fixed, set of $n$ self-adjoint boundary conditions

$$[u\alpha_{\delta j}](\tilde{b}) - [u\alpha_{\delta j}](\tilde{a}) = 0, \qquad (j = 1, \ldots, n). \tag{4}$$

Let $\mathfrak{D}_\delta$ be the set of all $u \in \mathfrak{H}$ having the properties: (i) $u$ has continuous derivatives up to the $(n-1)$st order on $\delta$, (ii) $u^{(n-1)}$ is absolutely continuous on $\delta$, (iii) $Lu$ is square summable on $\delta$, (iv) $u$ satisfies the boundary conditions (4). Define $H_\delta$ to be the operator with domain $\mathfrak{D}_\delta$ given by

$$\begin{aligned} H_\delta u(x) &= Lu(x), \qquad (x \in \delta), \\ &= 0, \qquad\quad (x \in' \delta). \end{aligned}$$

Then $H_\delta$ is self-adjoint, and we have the following result.

THEOREM 3. *If the spectral resolutions of $H$ and $H_\delta$ are given by*

$$H = \int_{-\infty}^{\infty} \lambda \, dE(\lambda), \qquad H_\delta = \int_{-\infty}^{\infty} \lambda \, dE_\delta(\lambda),$$

*then*

$$E_\delta(\lambda) \to E(\lambda), \qquad (\delta \to (a, b)),$$

*if $\lambda$ is not an eigenvalue of $H$.*

The convergence is meant in the pointwise sense on $\mathfrak{H}$. The proof results by showing first that the closure of the pointwise limit of the $H_\delta$ is just $H$, and then applying a theorem due to Rellich.[11] We remark that, with proper modifications, this theorem is valid for self-adjoint extensions $H$ of $T_0$ which occur in the case where either $a$ or $b$ is finite, and the domain of $H$ can be prescribed by a set of boundary conditions at the finite endpoint of $(a, b)$ alone. For example, in the Weyl case, this would occur on $(0, \infty)$ when $L$ is of the *limit circle* type at 0 and the limit point type at $\infty$.

The restriction of $H_\delta$ to the subspace $\mathfrak{H}_\delta$ of all square summable functions on $\delta$ is a self-adjoint operator with a discrete spectrum. Let $\{\chi_k\}$ be a complete orthonormal set of eigenfunctions, and $\{\lambda_k\}$ the corresponding eigenvalues. If $\Delta = (\mu, \lambda]$ is a finite $\lambda$-interval, and $E_\delta(\Delta) = E_\delta(\lambda) - E_\delta(\mu)$, then for $u \in \mathfrak{H}_\delta$,

$$E_\delta(\Delta)u = \sum_{\lambda_k \in \Delta} \left(\int_\delta u\bar{\chi}_k\right)\chi_k. \tag{5}$$

Suppose $s_j = s_j(x, l)$ $(j = 1, \ldots, n)$ is a set of $n$ linearly independent solutions of the equation $Lu = lu$, where $l$ is a complex number, and assume that these functions are chosen so that $d^m s_j(x, l)/dx^m$ $(j = 1, \ldots, n;$ $m = 0, 1, \ldots, n-1)$ are entire in $l$. In terms of such a set of functions

$$\chi_k(x) = \sum_{j=1}^{n} r_{kj} s_j(x, \lambda_k), \tag{6}$$

where the $r_{kj}$ are complex constants, depending on $\delta$. Placing (6) into (5), we can rewrite (5) in the form

$$E_\delta(\Delta)u(x) = \int_\delta E_\delta(x, y, \Delta)u(y)dy, \qquad (u \in \mathfrak{H}_\delta),$$

where the kernel $E_\delta(x, y, \Delta)$ is given by

$$E_\delta(x, y, \Delta) = \int_\Delta \sum_{j,k=1}^{n} s_j(x, \lambda)\bar{s}_k(y, \lambda)d\rho_{\delta jk}(\lambda).$$

Here the matrix $\rho_\delta = (\rho_{\delta jk})$ consists of step functions, and is hermitian, non-decreasing in the sense that $\rho_\delta(\Delta) = \rho_\delta(\lambda) - \rho_\delta(\mu)$ is positive semi-definite if $\lambda > \mu$, and the total variation of $\rho_{\delta jk}$ is finite on every finite $\lambda$-interval. It can be assumed that $\rho_\delta$ is normalized so that $\rho_\delta(0) = 0$ and $\rho_\delta(\lambda + 0) = \rho_\delta(\lambda)$.

THEOREM 4. *There exists an hermitian, non-decreasing matrix $\rho = \rho(\lambda)$, with elements of bounded variation on every finite $\lambda$-interval, such that if $u \in \mathfrak{H}$ is any continuous function vanishing outside some closed bounded subinterval of $(a, b)$, then*

$$E(\Delta)u(x) = \int^b E(x, y, \Delta)u(y)dy,$$

*where*

$$E(x, y, \Delta) = \int_\Delta \sum_{j,k=1}^{n} s_j(x, \lambda)\bar{s}_k(y, \lambda)d\rho_{jk}(\lambda).$$

*Further, this matrix is essentially unique, in the sense that*

$$\rho_{\delta jk}(\Delta) \to \rho_{jk}(\Delta), \qquad (\delta \to (a, b)), \tag{7}$$

*if the end-points of $\Delta$ are not eigenvalues of $H$.*

The existence of at least one limit matrix follows by an adaptation of a lemma due to Levitan.[8] The remainder of the theorem is a consequence of Theorem 3 and the fact that the spectral family of projections $E(\lambda)$ is unique.

We note that the limiting relation (7) is independent of the particular self-adjoint boundary conditions imposed on the finite interval $\delta$. Using a different approach Levinson showed in the case $n = 2$, $p_k$ real, that such a limiting relation held independent of *separated* boundary conditions on $\delta$.[5]

Using standard devices it is now possible to prove the Parseval equality and the expansion theorem. Following Kodaira,[7] we introduce vector functions of $\lambda$, $\varphi = (\varphi_1, \ldots, \varphi_n)$, $\psi = (\psi_1, \ldots, \psi_n)$ and the inner product

$$(\varphi, \psi) = \int_{-\infty}^{\infty} \sum_{j,k=1}^{n} \varphi_k(\lambda)\bar{\psi}_j(\lambda)d\rho_{jk}(\lambda).$$

Since $\rho$ is non-decreasing, $(\varphi, \varphi) \geqq 0$, and we can define a norm $\| \quad \|^*$ by $\|\varphi\|^* = (\varphi, \varphi)^{1/2}$. Let $\mathfrak{H}^*$ be the Hilbert space of all vectors $\varphi$ such that $\|\varphi\|^* < \infty$. Denote the norm in $\mathfrak{H}$ by $\| \quad \|$.

THEOREM 5. (*Parseval Equality and Expansion Theorem.*) *If $u \in \mathfrak{H}$ the vector $\varphi = (\varphi_j)$, where*

$$\varphi_j(\lambda) = \int_a^b \bar{s}_j(x, \lambda)u(x)dx,$$

*converges in norm in* $\mathfrak{H}^*$, *and*

$$\|\varphi\|^* = \|u\|.$$

*In terms of this* $\varphi$,

$$u(x) = \int_{-\infty}^{\infty} \sum_{j,k=1}^{n} s_j(x, \lambda)\varphi_k(\lambda)d\rho_{jk}(\lambda),$$

*where the integral converges in norm in* $\mathfrak{H}$.

* This work was supported in part by the Office of Naval Research.

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# ON THE MULTIPLICATION OF REPRESENTATIONS OF THE LINEAR GROUP

BY F. D. MURNAGHAN

INSTITUTO TECNOLÓGICO DE AERONÁUTICA, SÃO JOSÉ DOS CAMPOS, BRASIL

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We gave[1] several years ago a recurrence method for obtaining the analysis into its irreducible components of the Kronecker product of irreducible representations of the $n$-dimensional linear group and we provided tables furnishing the analysis of $\{\lambda\}\{\mu\}$, where $\{\lambda\}$ and $\{\mu\}$ are irreducible representations of degrees $p$ and $q$, respectively, for all values of $p$ and $q$ for which $p + q \leqslant 10$. Recently the problem has been investigated again in a useful paper by Newell[2] and we propose to show in the present note how greatly Newell's developments facilitate the provision of tables for which $p + q > 10$.

Since multiplication of $\{\lambda\}$ by $\{1\}$, by $\{2\}$ and by $\{1^2\}$ is simple and well known (for any $\{\lambda\}$) we illustrate the method by considering the problem of analyzing the product $\{\lambda\}\{21\}$. Our original method was based on a formula $\{21\} = z_1^2\{1\}' + z_1[\{2\}' + \{1^2\}'] + \{21\}'$ where the primed parentheses are functions of the $n - 1$ variables $z_2, \ldots, z_n$. If $(\lambda) = (\lambda_1)$ is a one-element partition of $p$ we obtain the product $\{\lambda_1\}\{21\}$ by applying to $\{\lambda_1\}$ the operator $\xi_1^2\{1\} + \xi_1[\{2\} + \{1^2\}] + \{21\}$where $\xi_1$ increases $\lambda_1$ by 1; in other words the product $\{\lambda_1\}\{21\}$ is furnished, *for all values of* $\lambda_1$, by $\{\lambda_1 + 2,1\} + \{\lambda_1 + 1,2\} + \{\lambda_1 + 1,1^2\} + \{\lambda_1,21\}$. If $\{\lambda_1\} = \{0\}$ is the identity representation this formula yields $\{21\} + \{12\} + \{1^3\} + \{021\}$ which reduces (as it must) to $\{21\}$. We may regard the formula $\{0\}\{21\} = \{21\} + \{12\} + \{1^3\} + \{021\}$ as a master-formula which yields the product of $\{21\}$ by any one-element-partition representation $\{\lambda_1\}$ by merely applying to it the operator $\xi_1^{\lambda_1}$, $\xi_1$ being an operator which increases by one the first element of any $\{\ldots\}$. For example, $\{8\}\{21\} = \{10.1\} + \{92\} + \{91^2\} + \{821\}$. When we pass to 2-element partitions $(\lambda_1, \lambda_2)$ the method originally used by us was to apply the operator $\xi_1^2\{1\}'\{\lambda_2\}' + \xi_1[\{2\}'\{\lambda_2\}' + \{1^2\}'\{\lambda_2\}'] + \{21\}'\{\lambda_2\}'$ to $\{\lambda_1, \lambda_2\}$ so that this method necessitated prior knowledge of the analyses involved in the coefficients of this quadratic operator. Newell's improvement consists in the provision of a master-formula furnishing (without preliminary calculations) the product $\{\lambda\}\{21\}$ where $(\lambda)$ is any 2-element partition of $p$ and, indeed, the provision of a whole series of such formulas, one for each value of $k$, $k$ being the number of elements in $(\lambda)$. These formulas become very elaborate as $k$ increases and it seems better to restrict our use of the master-formulas to the cases $k = 1, 2, 3$ and to revert to the recurrence method (applied to the formula corresponding to $k = 3$) when the number of elements in $(\lambda) >$

3. To obtain the master-formula for $\{21\}$, corresponding to $k = 2$, we use the relation $\{21\} = (z_1^2z_2 + z_1z_2^2) + (z_1^2 + 2z_1z_2 + z_2^2)\{1\}' + (z_1 + z_2)[\{2\}' + \{1^2\}'] + \{21\}'$ where, now, the primed parentheses are functions of the $n - 2$ variables $z_3, \ldots, z_n$. If $(\lambda) = (\lambda_1, \lambda_2)$ is any 2-element partition of $p$ we obtain $\{\lambda\}\{21\}$ by applying to $\{\lambda_1, \lambda_2\}$ the operator $(\xi_1^2\xi_2 + \xi_1\xi_2^2) + (\xi_1^2 + 2\xi_1\xi_2 + \xi_2^2)\{1\} + (\xi_1 + \xi_2)[\{2\} + \{1^2\}] + \{21\}$ where $\xi_1$ increases $\lambda_1$, and $\xi_2$ increases $\lambda_2$, by 1. The master-formula is $\{0\}\{21\} = \{21\} + \{201\} + \{12\} + 2\{1^3\} + \{102\} + \{101^2\} + \{021\} + \{012\} + \{01^3\} + \{0^221\}$; it yields $\{\lambda_1, \lambda_2\}\{21\}$ by merely applying to it the operator $\xi_1^{\lambda_1}\xi_2^{\lambda_2}$. For example $\{53\}\{21\} = \{74\} + \{731\} + \{65\} + 2\{641\} + \{632\} + \{631^2\} + \{5^21\} + \{542\} + \{541^2\} + \{5321\}$. The master-formula for $\{21\}$, corresponding to $k = 3$, is $\{0\}\{21\} = \{21\} + \{201\} + \{20^21\} + \{12\} + 2\{1^3\} + 2\{1^301\} + \{102\} + 2\{101^2\} + \{10^22\} + \{10^21^2\} + \{0^221\} + \{0201\} + \{012\} + 2\{01^3\} + \{0102\} + \{0101^2\} + \{0^221\} + \{0^212\} + \{0^21^3\} + \{0^321\}$; we obtain $\{\lambda\}\{21\}$, where $\{\lambda\}$ is any 3-element partition of $p$, by merely applying to it the operator $\xi_1^{\lambda_1}\xi_2^{\lambda_2}\xi_3^{\lambda_3}$. For example $\{431\}\{21\} = \{641\} + \{632\} + \{631^2\} + \{5^21\} + 2\{542\} + 2\{541^2\} + \{53^2\} + 2\{5321\} + \{531^3\} + \{4^23\} + 2\{4321\} + \{4^21^3\} + \{43^21\} + \{432^2\} + \{4321^2\}$. In making the table for $p + q = 11$ these formulas take care of all products $\{\lambda\}\{21\}$ save $\{51^3\}\{21\}$ and $\{421^2\}\{21\}$ since $\{\lambda\}\{21\}$ is the associate of $\{\lambda^*\}\{21\}$, $(\lambda^*)$ being the associate partition to $(\lambda)$. To obtain $\{51^3\}\{21\}$ we add 1 to the terms $\{721\} + \{631\} + \{62^2\} + \{532\}$ of the analysis of $\{51^2\}\{21\}$; we replace the 1 at the end of the terms $\{71^3\} + 2\{621^2\} + \{531^2\} + \{52^21\}$ by $\{2\} + \{1^2\}$; we replace the $\{2\} + \{1^2\}$ which follows each of the symbols $\{61^2\}$, $\{521\}$, by $\{3\} + 2\{21\} + \{1^3\}$ and we follow $\{51^2\}$ with $\{31\} + \{2^2\} + \{21^2\}$. Thus $\{51^3\}\{21\} = \{721^2\} + \{71^4\} + \{631^2\} + \{62^21\} + 2\{621^3\} + \{61^5\} + \{5321\} + \{531^3\} + \{52^21^2\} + \{521^4\}$. Similarly $\{421^2\}\{21\} = \{631^2\} + \{62^21\} + \{621^3\} + \{541^2\} + 2\{5321\} + 2\{531^3\} + \{52^3\} + 2\{52^21^2\} + \{521^4\} + \{4^221\} + \{43^21\} + \{4^21^3\} + \{432^2\} + 2\{4321^2\} + \{431^4\} + \{42^31\} + \{42^21^3\}$.

We give the master-formulas corresponding to $k = 1$ and $k = 2$, respectively, for a number of partitions $(\mu)$:

$k = 1$:

$\{1\}:\{1\} + \{01\}$; $\{1^2\}:\{1^2\} + \{01^2\}$; $\{2\}:\{2\} + \{1^2\} + \{02\}$; $\{1^3\}:\{1^3\} + \{01^3\}$; $\{21\}:\{21\} + \{12\} + \{1^3\} + \{021\}$; $\{3\}:\{3\} + \{21\} + \{12\} + \{03\}$; $\{1^4\}:\{1^4\} + \{01^3\}$; $\{21^2\}:\{21^2\} + \{121\} + \{1^4\} + \{021^2\}$; $\{2^2\}:\{2^2\} + \{121\} + \{02^2\}$; $\{31\}:\{31\} + \{2^2\} + \{21^2\} + \{13\} + \{121\} + \{031\}$; $\{4\}:\{4\} + \{31\} + \{2^2\} + \{13\} + \{04\}$; $\{1^5\}:\{1^5\} + \{01^5\}$; $\{21^3\}:\{21^3\} + \{121^2\} + \{1^5\} + \{021^3\}$; $\{2^21\}:\{2^21\} + \{12^2\} + \{121^2\} + \{02^21\}$; $\{31^2\}:\{31^2\} + \{2^21\} + \{21^3\} + \{131\} + \{121^2\} + \{031^2\}$;$\{32\}:\{32\} + \{23\} + \{2^21\} + \{131\} + \{12^2\} + \{03\ 2\}$

$\{41\}:\{41\} + \{32\} + \{31^2\} + \{23\} + \{2^21\} + \{14\} + \{131\} + \{041\}$; $\{5\}:\{5\} + \{41\} + \{32\} + \{23\} + \{14\} + \{05\}$.

$k = 2$:

$\{1\}:\{1\} + \{01\} + \{0^21\}$; $\{1^2\}:\{1^2\} + \{101\} + \{01^2\} + \{0^21^2\}$; $\{2\}:\{2\} + \{1^2\} + \{101\} + \{02\} + \{01^2\} + \{0^22\}$; $\{1^3\}:\{1^3\} + \{101^2\} + \{01^3\} + \{0^21^3\}$; $\{21\}:\{21\} + \{201\} + \{12\} + 2\{1^3\} + \{102\} + \{101^2\} + \{021\} + \{012\} + \{01^3\} + \{0^221\}$; $\{3\}:\{3\} + \{21\} + \{201\} + \{12\} + \{1^3\} + \{102\} + \{03\} + \{021\} + \{012\} + \{0^23\}$; $\{1^4\}:\{1^4\} + \{101^3\} + \{01^4\} + \{0^21^4\}$; $\{21^2\}:\{21^2\} + \{201^2\} + \{121\} + \{1^22\} + 2\{1^4\} + \{1021\} + \{101^3\} + \{021^2\} + \{0121\} + \{01^4\} + \{0^221^2\}$; $\{2^2\}:\{2^2\} + \{21^2\} + \{202\} + \{121\} + \{1^22\} + \{1^4\} + \{1021\} + \{02^2\} + \{0121\} + \{0^22^2\}$; $\{31\}:\{31\} + \{301\} + \{2^2\} + 2\{21^2\} + \{202\} + \{201^2\} + \{13\} + 2\{121\} + 2\{1^22\} + \{1^4\} + \{103\} + \{1021\} + \{031\} + \{02^2\} + \{021^2\} + \{013\} + \{0121\} + \{0^231\}:\{4\}:\{4\} + \{31\} + \{301\} + \{2^2\} + \{21^2\} + \{202\} + \{13\} + \{121\} + \{1^22\} + \{103\} + \{04\} + \{031\} + \{02^2\} + \{013\} + \{0^24\}$; $\{1^5\}:\{1^5\} + \{101^4\} + \{01^5\} + \{0^21^5\}:\{21^3\}:\{21^3\} + \{201^3\} + \{121^2\} + \{1^221\} + 2\{1^5\} + \{1021^2\} + \{101^4\} + \{021^3\} + \{0121^2\} + \{01^5\} + \{0^221^3\}$; $\{2^21\}:\{2^21\} + \{212\} + \{21^3\} + \{2021\} + \{12^2\} + \{121^2\} + 2\{1^221\} + \{1^5\} + \{102^2\} + \{1021^2\} + \{02^21\} + \{012^2\} + \{0121^2\} + \{0^22^21\}$; $\{32\}:\{32\} + \{31^2\} + \{302\} + \{23\} + 2\{2^21\} + 2\{212\} + \{21^3\} + \{203\} + \{2021\} + \{131\} + 2\{12^2\} + \{121^2\} + \{1^23\} + 2\{1^221\} + \{1031\} + \{102^2\} + \{032\} + \{023\} + \{02^21\} + \{0131\} + \{012^2\} + \{0^232\}$; $\{31^2\}:\{31^2\} + \{301^2\} + \{2^21\} + \{212\} + 2\{21^3\} + \{2021\} + \{201^3\} + \{131\} + \{12^2\} + 2\{121^2\} + \{1^23\} + 2\{1^221\} + \{1^5\} + \{1031\} + \{1021^2\} + \{031^2\} + \{02^21\} + \{021^3\} + \{0131\} + \{0121^2\} + \{0^231^2\}$; $\{41\}:\{41\} + \{401\} + \{32\} + 2\{31^2\} + \{302\} + \{301^2\} + \{23\} + 2\{2^21\} + 2\{212\} + \{21^3\} + \{203\} + \{2021\} + \{14\} + 2\{131\} + 2\{12^2\} + \{121^2\} + 2\{1^23\} + \{1^221\} + \{104\} + \{1031\} + \{041\} + \{032\} + \{031^2\} + \{023\} + \{02^21\} + \{014\} + \{0131\} + \{0^241\}$; $\{5\}:\{5\} + \{41\} + \{401\} + \{32\} + \{31^2\} + \{302\} + \{23\} + \{2^21\} + \{212\} + \{203\} + \{14\} + \{131\} + \{12^2\} + \{1^23\} + \{104\} + \{05\} + \{041\} + \{032\} + \{023\} + \{014\} + \{0^25\}$.

These formulas yield $\{\lambda\}\{\mu\}$ where $(\mu)$ is any partition of any integer $q \leqslant 5$ and $(\lambda)$, or $(\lambda^*)$, is any 1- or 2-element partition of any number $p$ whatever. For example, $\{9\}\{2^21\}$, obtained by applying the operator $\xi_1^9$ to the formula for $\{2^21\}$, $k = 1$, is $\{11.21\} + \{10.2^2\} + \{10.21^2\} + \{10.2^21\}$ and $\{83\}\{21^3\}$, obtained by applying the operator $\xi_1^8\xi_2^3$ to the formula for $\{21^3\}$, $k = 2$, is $\{10.41^2\} + \{10.31^3\} + \{951^2\} + \{9421\} + 2\{941^3\} + \{9321^2\} + \{931^4\} + \{851^3\} + \{8421^2\} + \{841^4\} + \{8321^3\}$. $\{1^p\}\{\mu\}$ is the associate of $\{p\}\{\mu^*\}$ and $\{2^{\lambda_2}1^{\lambda_1 - \lambda_2}\}\{\mu\}$ is the associate of $\{\lambda_1, \lambda_2\}\{\mu^*\}$. The manner in which the formulas for $k = 2$ were constructed will be sufficiently clear if we examine the formula for any one of them, say $\{41\}$. The terms beginning with 4 in this formula are obtained

by prefixing 4 to each term of the formula for $\{1\}$, $k = 1$; the terms beginning with 3 are obtained by prefixing 3 to each term of the sum of the formulas for $\{2\}$ and $\{1^2\}$, $k = 1, (\{1\}^2 = \{2\} + \{1^2\})$; the terms beginning with 2 are obtained by prefixing 2 to each term of the sum of the formulas for $\{3\}$ and $\{21\}$, $k = 1, (\{2\}\{1\} = \{3\} + \{21\})$; the terms beginning with 1 are obtained by prefixing 1 to each term in the sum of the formulas for $\{4\} + \{31\}$, $k = 1, (\{3\}\{1\} = \{4\} + \{31\})$ and the terms beginning with 0 are obtained by prefixing 0 to each term of the formula for $\{41\}$, $k = 1$.

We conclude with the remark that the master-formulas for $k = 3$ may be readily derived from those given above (for $k = 2$). Thus the terms beginning with 3 in the master-formula for $\{31^2\}$, $k = 3$, are obtained by prefixing 3 to the terms of the master-formula for $\{1^2\}$, $k = 2$; the terms beginning with 2 are obtained by prefixing 2 to the terms of the sum of the master-formulas for $\{21\}$ and $\{1^3\}$, $k = 2$; the terms beginning with 1 are obtained by prefixing 1 to the terms of the sum of the master-formulas for $\{31\}$ and $\{21^2\}$, $k = 2$; and, finally, the terms beginning with 0 are obtained by prefixing 0 to the terms of the master-formula for $\{31^2\}$, $k = 2$.

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## *THE DIRICHLET AND VIBRATION PROBLEMS FOR LINEAR ELLIPTIC DIFFERENTIAL EQUATIONS OF ARBITRARY ORDER*

BY FELIX E. BROWDER

DEPARTMENT OF MATHEMATICS, BOSTON UNIVERSITY

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Let $K$ be a linear elliptic differential operator of order $2m$ on a bounded domain $D$ of Euclidean $n$-space. In a previous note[1] the author presented a new method relating the existence and uniqueness of the solution of the Dirichlet problem for $K$ on $D$. Applying an extension of that method, we have established semi-boundedness for the general linear elliptic operator, the completeness of the eigenfunctions of the general self-adjoint elliptic operator, the existence of a fundamental solution in the large as well as of the Green's function for domains on which the Dirichlet problem has a unique solution, and the existence and properties of a kernel function representation of the solution of the Dirichlet problem for strongly positive linear elliptic equations. In addition the results of our previous note on the Dirichlet problem are extended to all unbounded domains whose complement has a non-empty interior, with a restriction only upon the

behavior of the coefficients of the non-positive part of the differential operator at infinity.

Throughout the following discussion, we shall use the notation of our previous note.

1. The suitably differentiable linear differential operator $K$ of order $2m$ is said to be positive if there exists $c > 0$ for which $(-1)^m \int_D Kf \cdot f \, dx \geq c(f,f)_m$, $f \in C_c^{2m}(D)$.[2] $K$ is said to be semi-bounded provided there exists a real number $d$ for which

$$(-1)^m \int_D Kf \cdot f \, dx \geq d(f,f)_0, \qquad f \in C_c^{2m}(D).$$

LEMMA 1. *Suppose that $K_1$ is positive and of order $2m$, $A$ suitably differentiable and of lower order, $K = K_1 + A$. Then $K$ is semi-bounded.*

*Proof:* It may be shown that there exist constants $c_1, c_2, c_3 > 0$ for which all $f \in C_c^{2m}(D)$

(1): $(-1)^m \int_D Kf \cdot f \, dx \geq c_1 \|f\|_m^2 - c_2 \|f\|_m \|f\|_{m-1}$

(2): $|(-1)^m \int_D Kf \cdot f \, dx| \leq c_3 \|f\|_m^2$.

We shall show that (1) and (2) imply that $K$ is semi-bounded.

Suppose that there exists a sequence $\{f_r\}$, $f_r \in C_c^{2m}(D)$, for which, if $k_r \int_D f_r^2 \, dx = (-1)^m \int_D Kf_r \cdot f_r \, dx$, then $k_r \to -\infty$ as $r$ goes to infinity. We may assume that $\|f_r\|_m = 1$ for all $r$. It follows from (1) and the condition upon the $k_r$ that there exists $\alpha > 0$ such that $\|f_r\|_{m-1} > \alpha$. Let $H_{m-1}$ be the completion of $C_c^{2m}(D)$ with respect to the $m-1$ norm. Then the natural mapping of $C_c^{2m}(D)$ into $L^2(D)$ may be extended to a continuous one-one mapping of $H_{m-1}$ onto a linear subset of $L^2(D)$.

By the Rellich Selection Theorem[3] there exists a subsequence $\{f_{r_i}\}$ of the sequence $\{f_r\}$ converging in $m-1$ norm to an element $u$ of $H_{m-1}$. Let $u$ also denote the element of $L^2(D)$ corresponding to the element $u$ of $H_{m-1}$. By (2), $|k_{r_i} \int_D f_{r_i}^2 \, dx| \leq c_3$. Since $|k_{r_i}|$ goes to infinity with $i$, $\int_D u^2 \, dx = \lim_i \int_D f_{r_i}^2 \, dx = 0$ which implies that $u = 0$. Thus $\|f_{r_i}\|_{m-1}$ goes to zero as $i$ goes to infinity, contradicting the inequality $\|f_r\|_{m-1} > \alpha > 0$. Q. E. D.

The suitably differentiable linear differential operator $K$ on $D$ is said to be elliptic if

$$K = \sum_{i_1, \ldots, i_{2m}} p_{i_1 \ldots i_{2m}}(x) \frac{\partial^{2m}}{\partial x_{i_1} \ldots \partial x_{i_{2m}}} + R$$

where $R$ is of order less than $2m$ and there exists $\rho > 0$ such that

$$\sum_{i_1, \ldots, i_{2m}} p_{i_1 \ldots i_{2m}}(x) \xi_{i_1} \ldots \xi_{i_{2m}} \geq \rho \sum_{i=1}^{n} \xi_i^{2m}$$

for all $x$ in the closure of $D$.[4]

THEOREM 1. *Let $K$ be a linear elliptic differential operator of order $2m$ on the bounded domain $D$ of $E^n$. Then there exists a real $d$ such that $K + d$ is positive and therefore $K$ is semi-bounded.*

*Proof:* Let

$$L = \sum_{i_1 \cdots i_{2m}} \frac{\partial^m}{\partial x_{i_1} \cdots \partial x_{i_m}} \left( p_{i_1 \cdots i_{2m}}(x) \frac{\partial^m}{\partial x_{i_{m+1}} \cdots \partial x_{i_{2m}}} \right).$$

Since $K = L + A$ where $A$ is of lower order, it follows from Lemma 1 that it is sufficient to prove the existence of a real number $d$ such that $L + d$ is positive. Since $L = (L - \epsilon \Delta^m) + \epsilon \Delta^m$, $\epsilon > 0$ ($\Delta$ = the Laplacian) and since $\epsilon \Delta^m$ is positive, it is only necessary to prove that $L - \epsilon \Delta^m$, which satisfies the same conditions as $L$ for $\epsilon$ sufficiently small, is semi-bounded. Thus we may restrict ourselves to the proof that $L$ is semi-bounded.

If the functions $p_{i_1 \cdots i_{2m}}$ were constants on $D$, it would follow from the Parseval equality for the $n$-dimensional Fourier transform that $(f, f) \geq \rho(f, f)_m$ for $f \in C_c^{2m}(D)$, where we set

$$(f, f) = (-1)^m \int_D Lf \cdot f \, dx =$$

$$\int_D \left\{ \sum_{i_1 \cdots i_{2m}} p_{i_1 \cdots i_{2m}}(x) \frac{\partial^m f}{\partial x_{i_1} \cdots \partial x_{i_m}} \frac{\partial^m f}{\partial x_{i_{m+1}} \cdots \partial x_{i_{2m}}} \right\} dx.$$

In the general case it follows from the uniform continuity of the functions $p_{i_1 \cdots i_{2m}}$ and the Schwarz inequality that there exists $\delta > 0$ such that if $f \in C_c^{2m}(D)$ and the diameter of the support of $f$ is less than $\delta$, then $(f, f) \geq \frac{\rho}{2}(f, f)_m$. Since $D$ is bounded there exists a finite open covering $\pi$ of $D$, each of whose elements is of diameter less than $\delta$. Let the family of functions $\{\eta_i(x)\}$ be an infinitely differentiable partition of unity corresponding to the covering $\pi$. We may assume that each of the functions $+\sqrt{\eta_i(x)}$ is also infinitely differentiable. Since $\{\eta_i(x)\}$ is a partition of unity, for $f \in C_c^{2m}(D)$

$$(f, f) = \sum_i \int_D \eta_i(x) \left\{ \sum_{i_1 \cdots i_{2m}} p_{i_1 \cdots i_{2m}}(x) \frac{\partial^m f}{\partial x_{i_1} \cdots \partial x_{i_m}} \frac{\partial^m f}{\partial x_{i_{m+1}} \cdots \partial x_{i_{2m}}} \right\} dx.$$

It follows easily that there exists $\beta_1 > 0$ for which

$$(f, f) \geq \sum_i (\sqrt{\eta_i} f, \sqrt{\eta_i} f) - \beta_1 \|f\|_{m-1} \|f\|_m.$$

Since by assumption $\sqrt{\eta_i}$ is infinitely differentiable for each $i$ and the support of each $\sqrt{\eta_i} f$ is of diameter less than $\delta$,

$$(f, f) \geq \frac{\rho}{2} \sum_i (\sqrt{\eta_i} f, \sqrt{\eta_i} f)_m - \beta_1 \|f\|_{m-1} \|f\|_m.$$

Similarly there exists $\beta_2 > 0$ for which

$$\sum(\sqrt{\eta_t} f, \sqrt{\eta_t} f)_m \geq (f, f)_m - \beta_2 \|f\|_{m-1} \|f\|_m.$$

Combining these two inequalities, we obtain

$$(f, f) \geq \frac{\rho}{2} (f, f)_m - \left(\beta_1 + \frac{\rho}{2} \beta_2\right) \|f\|_{m-1} \|f\|_m.$$

Since on the other hand there exists $\beta_3 > 0$ for which

$$|(f, f)| \leq \beta_3 (f, f)_m$$

it follows from the proof of Lemma 1 that $L$ is semi-bounded. Q. E. D.

2. Let $\hat{C}^j(D)$, $\hat{C}_c^j(D)$ be the complex-valued analogs of $C^j(D)$, $C_c^j(D)$. Let $(f, g)_m$ be defined for all $f, g \in \hat{C}_c^m(D)$ by

$$(f, g)_m = \int_D \left\{ \sum_{i_1 \ldots i_m} \frac{\partial^m f}{\partial x_{i_1} \ldots \partial x_{i_m}} \overline{\frac{\partial^m g}{\partial x_{i_1} \ldots \partial x_{i_m}}} \right\} dx.$$

If $\hat{H}$ is the (complex) Hilbert space completion of $\hat{C}_c^m(D)$ with respect to this inner product, then the natural identification of $\hat{C}_c^m(D)$ with a subset of $\hat{L}^2(D)$ may be extended to a one-one identification of $\hat{H}$ with a subset of $\hat{L}^2(D)$. Let $B$ be a positive self adjoint suitably differentiable differential operator on $D$ of order less than $2m$. The function $u \in \hat{C}^{2m}(D)$ is said to be an eigenfunction of the differential operator $K$ of order $2m$ with respect to $B$ if $u \in \hat{H}$ and $Ku = \lambda Bu$ where the complex number $\lambda$ is the eigenvalue corresponding to $u$.

THEOREM 2. *Let $K$ be a linear elliptic differential operator of order $2m$ on the bounded domain $D$ of $E$, $B$ positive self adjoint on $D$ and of order less than $2m$. Then:*

*(a) The eigenvalues of $K$ with respect to $B$ are a discrete set of complex numbers and the set of eigenfunctions corresponding to any single eigenvalue is of finite linear dimension.*

*(b) Suppose $\lambda = \xi + i\zeta$ is an eigenvalue of $K$ with respect to $B$. Then there exists $\xi_0$ such that $(-1)^m \xi \geq (-1)^m \xi_0$ for all eigenvalues $\lambda$. Given any $\epsilon > 0$, $|\zeta| < \epsilon (-1)^m \xi$ except for a finite number of eigenvalues. If $K = L_1 + A_1$ where $L_1$ is self-adjoint and the order of $A_1$ is less than or equal to $m$, then there exists $R > 0$ such that $|\zeta|^2 \leqq (-1)^m R(\xi - \xi_0)$ for all eigenvalues $\lambda$.*[5]

*Proof:* The conclusions of (*a*) follow from the method of the author's previous note.[1] The conclusions of (*b*) follow from the method applied in the proof of Lemma 1 and Theorem 1.

THEOREM 3. *Let $K$ be a self-adjoint linear elliptic differential operator of order $2m$ on the bounded domain $D$ of $E^n$. Then the real eigenfunctions of $K$ with respect to $B$ are complete in $H_m$.*

*Proof:* Since $B$ is positive, by Theorem 1 there exists a real number $\lambda_0$ such that $K + \lambda_0 B$ is positive and self-adjoint. Let $(f, g) = (-1)^m \int_D (K + \lambda_0 B) f \cdot g \, dx$ for $f, g \in C_c^{2m}(D)$. Since $K$ and $B$ are suitably differentiable and $K + \lambda_0 B$ is positive, $(,)$ can be extended to an inner product on $H$ yielding a norm equivalent to the $m$-norm. By the argument of our preceding note, there exists a completely continuous linear transformation $U$ of $H$ defined by the condition that $(Uf, g) = \int_D Bf \cdot g \, dx$ for $f, g \in C_c^{2m}(D)$. It is easy to see by the self-adjointness of $B$ that $(Uf, g) = (f, Ug)$ for $f, g \in C_c^{2m}(D)$ and therefore $U$ is self-adjoint on $H$ with respect to the inner product $(,)$. From the argument of the author's previous note, it follows that for $u \in H$, $u \in C^{2m}(D)$ and $Ku = \lambda Bu$ if and only if

$$(-1)^m u = (\lambda + \lambda_0) Uu.$$

Moreover 0 is not an eigenvalue of $U$ because of the positiveness of $B$. The conclusion of Theorem 3 follows from the completeness of the eigenfunctions of a self-adjoint completely continuous linear operator on a real Hilbert space. Q. E. D.

3. If $K$ is a linear elliptic differential operator on the domain $D$ of $E^n$, the function $e(x, z)$ defined for $x, z$ in $D$, $x \neq z$, is said to be a fundamental solution for $K$ in $D$ if

$$f(x) = \int_D e(x, z) \cdot \overline{K} f(z) \, dz \qquad \text{for } f \in C_c^{2m}(D).$$

F. John[6] has shown the existence of a fundamental solution in the small for the general linear elliptic operator by using the Parametrix method of E. E. Levi together with results on fundamental solutions of equations with polynomial coefficients. Completing John's discussion for an arbitrary bounded domain by applying our results on the Dirichlet problem, we obtain the following theorem in the large.

THEOREM 4. *Let $K$ be a linear elliptic differential operator of order $2m$ on the bounded domain $D$ of $E^n$. Suppose that the Dirichlet problem for $K$ on $D_1$, a domain containing $D$, has only the zero solution for zero boundary values. Then there exists a fundamental solution $e(x, z)$ for $K$ on $D$ which is $2m$ times differentiable in $x$ and $z$ for $x \neq z$ and satisfies the equation $K_z e(x, z) = 0$ for $x \neq z$.*

4. As a by-product of the proof of Theorem 2 of the author's previous note,[7] we obtain the following:

THEOREM 5. (*a*) *Let $K$ be a linear elliptic differential operator on the domain $D$ of $E^n$. Let $N, N_1$ be neighborhoods of $x_0$ in $D$ with $N_1$ bounded such that the closure of $N_1$ is contained in $N$. Then there exists $M > 0$ such that if $u \in C^{2m}(N)$ is a solution of the equation $Ku = 0$, then $|u(x)|^2 \leq M \int_N u^2 \, dx$ for all $x \in N_1$.*

(*b*) *Let $\{u_i\}$ be a locally uniformly bounded family of solutions of the equation $Ku = 0$ where each $u_i \in C^{2m}(D)$. Then there exists a subsequence*

*$\{u_{ij}\}$ converging uniformly with all derivatives of order up to 2m in every compact subset of D to a solution $u_0 \in C^{2m}(D)$ of the equation $Ku = 0$.*

*(c) If the coefficients of K are infinitely differentiable in D then the conclusions of (a) hold for every partial derivative of u of any order. Under the hypotheses of (b) for such a K, the partial derivatives of the functions of the subsequence $\{u_{ij}\}$ converge uniformly on every compact subset of D.*

Part (a) of Theorem 5 is sufficient to carry through the standard argument[8] for the existence and properties of the kernel function with respect to the inner product (,) as well as the representation of the kernel function in terms of a complete orthonormal family of solutions, provided that $K$ satisfies certain restrictions. $K$ must be self-adjoint and the inner product (,) on $C_c^{2m}(D)$ must be the restriction of a positive inner product on $C^{2m}(D)$. The solution of the Dirichlet problem for such equations can be expressed in terms of the kernel function in Poincare domains[9] and with further positivity restrictions on $K$ in arbitrary bounded domains.

5. In this section we consider the Dirichlet problem for a linear elliptic operator $K$ on an unbounded domain $D$ in $E^n$ whose complement has at least one interior point.

Let $D_i$ be the subset of $D$ of all points whose distance from the origin is less than $i$. Let $S(D)$ be the complete metric space of functions in $D$ square-summable on every $D_i$ with the metric

$$d(f, g) = \sum_{i=1}^{\infty} \frac{1}{2^i} \frac{\|f - g\|_{o, D_i}}{1 + \|f - g\|_{o, D_i}}$$

Let $(f, g) = (-1)^m \int_D Kf \cdot g \, dx$; $f, g \in C_c^{2m}(D)$. If $K$ is a positive self-adjoint linear elliptic operator on $D$, and $H$ the Hilbert space which is the completion of $C_c^{2m}(D)$ with respect to (,), then the natural identification of $C_c^{2m}(D)$ with a subset of $S(D)$ may be extended to a one-one continuous mapping of $H$ onto a linear subset of $S(D)$. We shall identify $H$ with this subset.

If $g \in C^{2m}(D)$, $g$ is said to be a permissible boundary value function with respect to $K$ provided that there exists $k > 0$ for which $|(f, g)| \leq k\|f\|$ for $f \in C_c^{2m}(D)$.[10] If $K = L + A$, where $L$ is positive self-adjoint and $A$ is of order less than $2m$, the Dirichlet problem for $K$ on $D$ may be formulated as follows: Given $g$, a permissible boundary value function with respect to $L$, to find $u \in C^{2m}(D)$ such that $Ku = 0$ in $D$ and $u - g \in H$.

THEOREM 6. *(a) Let K be a linear elliptic operator which is positive self-adjoint. Then for every permissible boundary value function g, the Dirichlet problem has one and only one solution.*

*(b) Let $K = L + A$, where L is positive self-adjoint A of lower order. Suppose that the coefficients of A satisfy the following conditions at infinity:*

*(1) The coefficients of the* $r$th *derivatives for* $r \leq m$ *are* $O(1/r^{2m-r+\epsilon})$ *for some* $\epsilon > 0$.

*(2) For* $r > m$, $j \leq r - m$, *the* $j$th *derivatives of the coefficients of the* $r$th *derivative terms in* $A$ *are* $O(1/r^{2m+j-r+\epsilon})$ *for some* $\epsilon > 0$.

*Then if the Dirichlet problem for* $K$ *on* $D$ *with zero boundary values has only the zero solution, the Dirichlet problem for* $K$ *on* $D$ *has a solution for every boundary value function* $g$ *permissible with respect to* $L$.

[1] Browder, F. E., "The Dirichlet Problem for Linear Elliptic Equations of Arbitrary Even Order with Variable Coefficients," PROC. NATL. ACAD. SCI., **38,** 230–235 (1952).

[2] The definition of positivity used here is stronger than the usual form in $L^2(D)$.

[3] Courant-Hilbert, *Methoden der Mathematischen Physik*, Vol. II, Berlin, 1937; p. 489.

[4] Condition (*b*) for ellipticity of our previous note is superfluous since every elliptic differential operator can be written as the sum of a positive operator and one of lower order. This fact was observed by L. Gårding who has announced the semi-boundedness of the homogeneous operator $L$ defined in the proof of Theorem 1 as well as results on the Dirichlet problem for equations with infinitely differentiable coefficients similar to those of our previous note; *Compt. rend.*, **253,** 1554–1556 (1951).

[5] The results of (*b*) contain similar results obtained for the general second-order equation by Carleman, "Uber die asymptotische Verteilung der Eigenwerte partieller Differentialgleichungen," *Ber. Sachs, Akad.*, **88,** 128–129 (1936), Satz V.

[6] John, F., "General Properties of Solutions of Linear Elliptic Partial Differential Equations," Proceedings of the Symposium on Spectral Theory and Differential Problems, Stillwater, Okla. (1951), Chap. III.

[7] Cf. reference 1, p. 232.

[8] Bergman, S., "The Kernel Function and Conformal Mapping," Mathematical Survey Series, 1950.

[9] Courant-Hilbert, Vol. II, p. 511.

[10] A sufficient condition for $g$ to be permissible with respect to $L$ is that $|(f, g)| \leq K\|f\|_m$ for all $f \in C_c^{2m}(D)$.

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## *PRODUCTION OF PLAQUES IN MONOLAYER TISSUE CULTURES BY SINGLE PARTICLES OF AN ANIMAL VIRUS*

BY RENATO DULBECCO

CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

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Research on the growth characteristics and genetic properties of animal viruses has stood greatly in need of improved quantitative techniques, such as those used in the related field of bacteriophage studies.

The requirements for a quantitative virus technique are as follows: (1) The use of a uniform type of host cell; (2) an accurate assay technique; (3) the isolation of the progeny of a single virus particle; and (4) the separate isolation of each of the virus particles produced by a single infected

cell. In bacterial virus work, production of plaques by single virus particles on a uniform bacterial layer fulfills the first three requirements. The fourth requirement is easily met, in the case of bacteriophages.

In this article we shall show that plaques can similarly be produced by animal viruses and that their properties fulfill the first three requirements.

We have found that the virus of Western Equine Encephalomyelitis,

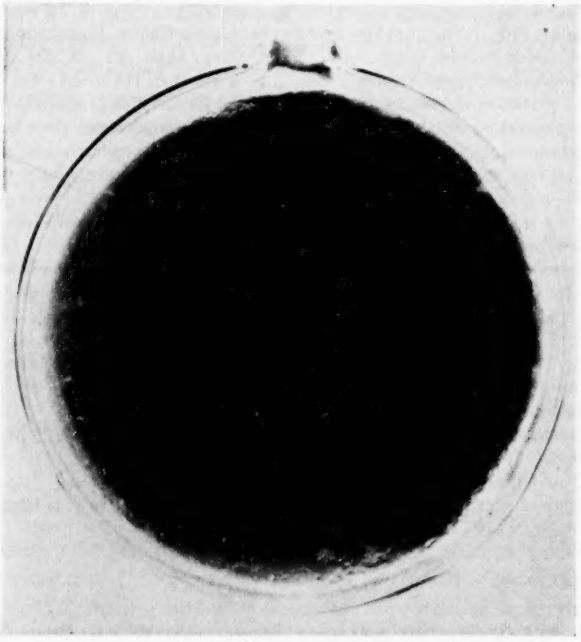

FIGURE 1

Plaques of Western Equine Encephalomyelitis virus on chicken fibroblasts. The plaques appear as round clear areas. The picture was taken against a distant dark background, sufficiently large to exactly cover the projection of the flask, surrounded by a uniformly brilliant light source, so as to detect the light scattered through a small angle by the cell debris in the plaques.

adapted to chicken embryo,[1] will produce plaques when it is grown on a monolayer of cells obtainable from chicken embryos by a modification of a technique devised by Shannon, Earle and Walts.[2] We use the following procedure: Under aseptic conditions, five 9-day-old chicken embryos are collected, decapitated, washed in Earle's saline solution (ES)[3] and pressed through a stainless steel, 24-mesh, wire cloth (supplied by Ludlow-Saylor

Wire Company, Los Angeles) which had been fitted at the bottom of a 50-ml. syringe. The resulting tissue pulp is collected in a 40-ml. centrifuge tube containing 15 ml. ES. After gravity sedimentation (10 minutes) the supernatant liquid containing cell debris and red blood cells is discarded and replaced with 15 ml. of a 0.5% solution of pure trypsin in ES. The tube is placed in a water bath at 37°C. for 10 minutes and then vigorously stirred by repeated pipetting with a 2-ml. automatic pipette. Owing to digestion of the connective fibers, a large fraction of the cells become free. The pieces of tissue which remain are eliminated by straining the suspension through the sieve unit described by Evans *et al.*[4] The cell suspension is then washed twice by centrifuging at 1000 r. p. m. for two minutes and resuspending the sediment in 15 ml. ES. The cell concentration in the final suspension is determined by counting in a hemocytometer.

An aliquot of the cell suspension containing $3 \times 10^7$ cells is introduced

TABLE 1

RELATIVE NUMBER OF PLAQUES PRODUCED BY WESTERN EQUINE ENCEPHALOMYELITIS VIRUS EXPOSED TO SPECIFIC ANTISERUM AND OTHER SUBSTANCES BEFORE INOCULATION INTO THE FLASKS

| TIME OF CONTACT WITH THE VIRUS, MIN. | TREATMENT | | | | |
|---|---|---|---|---|---|
| | DIST. WATER | ES | NORMAL HORSE SERUM 1:10 IN ES | HORSE SERUM HEATED AT 56°C. 30 MIN., 1:10 IN ES | HYPERIMMUNE HORSE SERUM HEATED AT 56°C. 30 MIN., 1:10 IN ES |
| 0 | 1 | 1 | 1 | 1 | 1 |
| 7 | .. | .. | .. | 0.92 | 0.15 |
| 10 | .. | .. | 0.63 | .. | .. |
| 15 | 1.1 | 0.8 | .. | 0.74 | 0.085 |
| 20 | .. | .. | 0.25 | .. | .. |
| 22 | .. | .. | .. | 0.67 | 0.055 |
| 30 | 0.9 | 1 | .. | .. | 0.01 |

into a large Pyrex Carrel flask (80 mm. inside diameter) which had been acid cleaned by the procedure described by Earle.[5] The volume is brought up to 5 ml. with ES. There are added 5 ml. of horse serum and 1 ml. of 1:1 chicken embryo extract. The flask is hermetically closed with a rubber stopper. Approximately two such flasks can be prepared from one embryo.

The flasks are incubated in horizontal position at 37°C.

During the first few hours of incubation the cells settle out and stick to the bottom of the flask singly or in small clumps. They start to grow very soon and within 48 hours produce a continuous cell layer which covers the bottom of the flask. The layer is mostly one cell thick and is formed by rather uniform spindle cells and by a small number of macrophages. The spindle cells are similar in morphology and rate of growth to the fibroblasts obtained by culturing the connective tissue of the chicken embryo; for this reason we called them fibroblasts. Other cell types carried with the

inoculum must obviously be present in the culture, but they are outnumbered by the fast-growing fibroblasts. A more uniform cell population can be obtained by using as inoculum a cell suspension obtained from the cell layer of one of these flasks by means of the described trypsin technique.

The relatively uniform cell layer is infected with the virus in the following way. The nutrient fluid is removed from the flask and the cell layer is washed with 5 ml. ES, which is then discarded. A virus sample of 0.5 ml. is introduced into the flask and uniformly spread on the cell layer by rocking. The flask is incubated for 30 minutes at 37°C. to allow attachment of of the virus to the cells, and the cell layer is then covered with a layer of

TABLE 2

NUMBER OF PLAQUES OBTAINED FROM STOCKS OF WESTERN EQUINE ENCEPHALOMYELITIS VIRUS AT DIFFERENT DILUTIONS

Experiments 1 and 2 were made with the same virus stock, experiments 3, 4 and 5 with a different stock. The virus consisted always of 20% extract of chicken embryo moribund after inoculation with the virus. Each flask was inoculated with 0.1 ml. of the diluted virus.

| NUMBER OF THE EXPERIMENT | DILUTION OF THE VIRUS | NUMBER OF PLAQUES ON EACH FLASK |
|---|---|---|
| 1 | $5 \times 10^3$ | 117; 180 |
| | $10^4$ | 65; 87 |
| 2 | $10^4$ | 59; 53 |
| | $3 \times 10^4$ | 28; 13; 18; 14; 19; 14; 18 |
| 3 | $10^3$ | 79 |
| | $3 \times 10^3$ | 23 |
| 4 | $10^3$ | 50 |
| | $4 \times 10^3$ | 12 |
| | $8 \times 10^3$ | 2 |
| 5 | $2 \times 10^3$ | 26 |
| | $6 \times 10^3$ | 10 |

5 ml. of melted agar containing nutrient fluid similar to that used for growing the cell layer. After solidification of the agar, the flasks are incubated for three days at 37°C. At this time they show round necrotic areas of 2 to 4 mm. in diameter, visible to the eye as bright areas against a dark background (Fig. 1). These areas are easily recognized under low-power magnification because the cells have been transformed into a granular debris that scatters the light. That these areas, which we call plaques, are produced by virus activity is shown by the fact that they are absent when the virus is absent and their number increases with the concentration of the virus. Prior treatment of the virus with specific antiserum reduces the number of plaques produced (table 1).

Each of these plaques is produced by one virus particle. This can be shown by determining the effect of dilution on the number of plaques produced by a given virus sample. The theoretical relation between the number of plaques and the virus concentration depends on the minimum number of virus particles required to produce a lesion. A linear dependence of the number of plaques on virus concentration can be obtained only if each plaque is produced by one virus particle. The results of several experiments show that this dependence is in fact linear, and so prove that one particle is sufficient to produce one plaque (table 2).

What fraction of the virus particles contained in a sample is able to produce plaques is not known. We have determined the relation between the number of particles able to produce a plaque under our conditions and the number of particles able to infect the chicken embryo by the standard inoculation procedure on the chorioallantoic membrane.[6] Two batches of 15 eggs each, incubated for 10 days, were inoculated with an amount of virus able to produce, respectively, 0.7 and 0.35 plaques on the average.

TABLE 3

COMPARISON OF THE TITER OF A SINGLE STOCK OF WESTERN EQUINE ENCEPHALOMYELITIS VIRUS BY PLAQUE COUNT AND EGG TITRATION

The plaque count titer is derived from Exp. 1, table 2 and equals $7.5 \times 10^6$/ml. The data of the egg titration are given here. Each egg was inoculated with 0.1 ml. of the virus dilution.

| BATCH OF EGGS | VIRUS DILUTION | SURVIVING EGG FRACTION | $-\log_2$ SURVIVING EGG FRACTION | TITER, PER ML. |
|---|---|---|---|---|
| 1 | $10^6$ | 0.16 | 1.8 | $1.8 \times 10^7$ |
| 2 | $2 \times 10^6$ | 0.50 | 0.7 | $1.4 \times 10^7$ |

The eggs were then incubated for 30 hours at 37°C., then scored for live and dead. The live ones were further incubated for 24 hours to check their vitality; all survived. From the fraction of live eggs in each batch the average number of infecting virus particles with which each egg was inoculated was determined by assuming a Poisson distribution of the particles per egg. This assumption is legitimate in view of the proof previously given in this article that infection of the cells is produced by one virus particle. The data of one such experiment, reported in table 3, show that the titer was approximately twice the titer obtained by plaque count—a difference which is not significant because of the large standard error of egg titration. In another experiment the egg titration gave a titer somewhat lower than the plaque count.

We conclude from these experiments that there is a nearly one-to-one ratio between the number of plaques and the number of infective virus particles. This result on the one hand indicates that the plaque count is an

efficient assay technique; on the other hand, it establishes a basic concept concerning animal virus action, namely, that infection of an embryo is produced by one virus particle.

The use of the plaque count as an assay technique has important practical advantages, as shown by the fact that the accuracy obtained with only one flask of this type is equal to that obtained with not less than one hundred chicken embryos in the currently used Reed and Muench technique.

We do not know whether many other viruses will exhibit this property of producing plaques under our conditions. So far, we have tried only one other virus, the Newcastle Disease virus,[7] and the results here were positive. The plaques formed by Newcastle Disease virus and those formed by Western Equine Encephalomyelitis virus are distinguishable under the microscope; the areas of cell destruction in the former are outlined by a halo of abnormally large cells or cell clumps of unknown nature, whereas in the latter there is seen only cell debris. The possibility of distinguishing the plaques produced by two different viruses on the same flask should be very valuable for the study of problems where two different viruses are involved, as in the phenomenon of interference.

*Summary.*—Plaque formation by single virus particles has been obtained with the virus of Western Equine Encephalomyelitis in a monolayer of chicken embryo fibroblasts grown *in vitro*. That a plaque is produced by a single virus particle is shown by the proportionality between number of plaques and virus concentration. The comparison between the number of plaques produced and the fraction of chicken embryos infected by the same virus sample indicates that nearly all virus particles able to infect the embryo produce a plaque; there is therefore a nearly 1:1 relation between infecting particles and plaques.

Plaques produced by this virus are distinguishable from the plaques produced by one other virus tested, the Newcastle Disease virus.

[1] Obtained from Dr. S. Lennette, California State Department of Public Health, Berkeley, Calif.

[2] Shannon, J. E., Earle, W. R., and Walts, H. K., *J. Natl. Cancer Inst.* (in press).

[3] Earle, W. R., *J. Nat. Cancer Inst.* **4**, 165–212 (1943).

[4] Evans, V. J., Earle, W. R., Sanford, K. K., and Shannon, J. E., *Ibid.*, **11**, 907–927 (1951).

[5] Earle, W. R., *Ibid.*, **4**, 131–133 (1943).

[6] Beveridge, W. I. B., and Burnet, F. M., "The Cultivation of Viruses and Rickettsiae in the Chick Embryo," His Majesty's Stationery Office, London, 1946, p. 14.

[7] Strain B, obtained from F. B. Bang, Johns Hopkins Medical School, Baltimore, Md

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